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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

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THE DETERMINATION OF SUGAR FERMENTATION OF BACTERIA

COMPARATIVE STUDIES

by

M. E. PARMALA

(Received for publication February 17, 1953)

The sugar fermentation is an essential criterion in the identification of bacteria. In routine tests, 0.5% sugar broth tubes are most commonly used. Some technics have been developed lately to conserve both the culture medium and sugars and to produce results without delay. Of these technics, the Spaur-Wynne's dry-disc-agar plate is economical when expensive sugars are studied, while Hannan-Weaver's technic and Clarke-Covan's enzymatic technics are above all rapid methods. An attempt has been made in the following to study the usefulness of these technics by testing the sugar-fermentation of bacterial strains with six different technics.

The technics used:

(1) Normal 0.5% sugar peptone broth tube with bromthymol-blue as the indicator — SB.

(2) Modified Spaur-Wynne's dry-disc-agar plate method (1), where the sugar-saturated, dried and sterilised discs are placed on the surface of an ordinary nutrient agar plate containing bromthymol-blue — DDP.

(3) Hannan-Weaver's rapid micromethod — RM (2).

(4) Clarke-Covan's enzymatic method in indicator buffer solution — BIS (3).

(5) Clarke-Covan's enzymatic method in agar tube — AT (3).

TABLE 1

COMPARISON OF RESULTS OBTAINED WITH THE DIFFERENT TECHNIQUES, GROUPED BY TYPES OF BACTERIA

		SB			DDP			RM			BIS			AT			AR		
		g	l	m	g	l	m	g	l	m	g	l	m	g	l	m	g	l	m
E. coli	+	6	6	6	5	2	2	6	1	6	6	—	6	3	1	2	5	5	5
	±	—	—	—	—	3	3	—	3	—	—	1	—	2	1	3	—	—	—
	—	—	—	—	—	—	—	—	2	—	—	5	—	—	3	—	—	—	—
Lact. neg. coli	+	4	—	3	4	—	1	4	—	3	4	—	4	2	—	1	4	—	3
	±	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	1	—
	—	—	4	1	—	4	3	—	4	—	—	4	—	—	2	—	—	3	1
Klebsiella	+	3	3	3	3	3	3	3	2	3	3	1	3	2	2	2	3	3	3
	±	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	1	—	—	1	—	—	—	—	—	—	—
Aerogenes	+	1	1	1	1	1	1	1	1	1	1	—	1	1	—	—	1	1	1
	±	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Proteus	+	6	—	—	6	—	2	4	—	1	6	—	6	3	—	5	6	—	1
	±	—	—	1	—	1	—	2	—	4	—	—	—	2	—	—	—	3	1
	—	—	6	5	—	5	4	—	6	1	—	6	—	—	5	—	—	3	4
Pseudomonas	+	3	—	1	2	—	—	1	—	—	2	—	—	3	—	—	5	—	—
	±	2	—	—	3	—	—	—	—	—	1	—	3	2	1	4	1	1	2
	—	2	7	6	1	6	6	6	7	7	4	7	4	1	5	2	—	5	4
Faecalis alkalig.	+	—	—	—															
	±	—	—	—	1	—	—	1	—	—	—	—	—				—	—	—
	—	1	1	1	—	1	1	—	1	1	1	1	1				1	1	1
Alkalesc. Dispar	+	1	1	—	1	1	—	1	1	—	1	—	1	1	—	—	1	1	—
	±	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—
	—	—	—	1	—	—	1	—	—	—	—	1	—	—	1	1	—	—	1
Gr.-neg. rods	+	9	5	6	9	4	3	9	3	7	9	1	8	4	1	4	7	6	6
	±	—	1	1	—	2	2	—	—	2	—	—	1	2	—	2	—	1	—
	—	—	3	2	—	3	4	—	6	—	—	8	—	—	5	—	1	1	2
Micrococ. v. aureus	+	5	3	4	4	4	4	2	—	1	2	—	2	3	1	2	2	3	2
	±	—	1	—	—	—	—	2	—	2	1	1	1	—	1	1	1	—	1
	—	—	1	1	—	—	—	1	5	2	2	4	2	—	1	—	1	1	1
Micrococ. v. albus	+	3	2	2	1	1	1	2	—	2	—	—	1	2	1	2	2	—	1
	±	1	—	1	1	—	—	—	—	1	1	—	—	1	—	1	1	2	2
	—	—	2	1	1	2	2	2	4	1	3	4	3	—	2	—	1	2	1
Enterococ.	+	1	1	1	1	1	1	1	1	1	1	1	1				1	1	1
	±	—	—	—	—	—	—	—	—	—	—	—	—				—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—				—	—	—

(6) Quantitative sugar determination with Dreywood's anthrone reagent — AR (4). The culture medium containing 0.5% sugar in peptone broth without indicator was diluted with distilled water 1: 80. 4 ml of anthrone reagent is added to 2 ml of the dilution in a 15 mm wide tube, mixed and allowed to stand for about 15 minutes, after the which the colour is determined by means of EEL photoelectric colorimeter.

All the tests were made with three different sugars: glucose, lactose and maltose. Of the bacterial strains under examination 6 were *E. coli*, 4 lactosenegative or »late lactose splitting» coli, 3 *Klebsiella*, 1 *Aerogenes*, 6 different bacteria of the *Proteus* group, 7 different bacteria of the *Pseudomonas* group, 1 *Faecalis alkaligenes*, 1 *Alkalescens-Dispar*, 9 unidentified gram-negative rods, 5 *Micrococcus pyogenes var. aureus*, 4 *Micrococcus pyogenes var. albus* and 1 *Enterococcus*.

The results can be seen in Table 1, where the values obtained from the different methods are presented by bacterial groups. In sugar broth tubes, in dry-discs-agar plate and in tubes for the anthrone method the results were read after 24 hours incubation, in the rapid micromethod and indicator buffer solution after 4 hours incubation and in agar tubes after 6 hours incubation. The interpretation of the results is indicated in Table 2.

TABLE 2

INTERPRETATION OF THE RESULTS OBTAINED BY THE DIFFERENT METHODS

	+	±	—
SB	completely yellow	yellowish green	no change of colour
DDP	a yellow zone of over 5 mm outside the disc or ring formation	a yellow zone of less than 5 mm outside the disc	neither zone nor ring
RM	whole tube yellow	about half of the tube yellow	no yellowness or only in the immediate vicinity of the bacterial mass
BIS	yellow	orange yellow	no change
AT	at least the upper half of the agar column yellow	distinct yellowness below the agar surface	no yellowness observable
AR	more than 10% of sugar disappeared	about 5—10% of sugar disappeared	no distinct fall in the sugar quantity

DISCUSSION

In the new technics (DDP, RM, BIS and AT) the danger of laboratory infection is almost eliminated. The short incubation time and large inoculum in microtechnics minimise contamination errors, insignificant in plate technics also.

The main advantages of the plate technic (DDP) are the easy preservation of the sugar-saturated blotting-paper discs and the minimal consumption of the expensive and hard-to-obtain sugars. The sterilisation of the culture medium is also simple. It is possible to perform 4 different sugar determinations with gram-negative rods by using the same plate. However, the extensive colour change zone of the indicator in gram-positive cocci limits this figure to two. The change of colour is not always clear with Proteus and Klebsiella strains but a positive result can be seen as a greenish ring consisting of a more generous growth about 5 mm outside the discs. A disadvantage of the plate method is that it fails to indicate the gas formation ability. In the original Spaur-Wynne method too the determination of the gas forming ability is uncertain and the method more inconvenient since the discs must be placed on the bottom of the plate before the culture is poured into the plate. This limits somewhat the use of the method for routine purposes.

Rapidity and the indication of gas formation are the advantages os Hannan-Waever's microtechnic (RM). Micro-tubes need not be sterilised, either, before use. The tubes are filled in a handy way by means of a Pasteur pipette, but agar addition increases the manipulations. Small tubes also are awkward to handle for result reading. The culture medium may become contaminated during storage.

Clarke-Covan's indicator buffer solution tube technic (BIS) is very easy to apply. Unsterilised tubes can be used and pipetting is readily effected with a micropipette. The results are all clear after 4 hours incubation. However, gas formation cannot be seen and the sugar solution in storage tubes is subject to easy contamination.

Clarke-Covan's agar tube technic (AT) is easier still. The tubes must be sterilized before agar is poured in but completed tubes keep well in a refrigerator. As with the former technic gas forma-

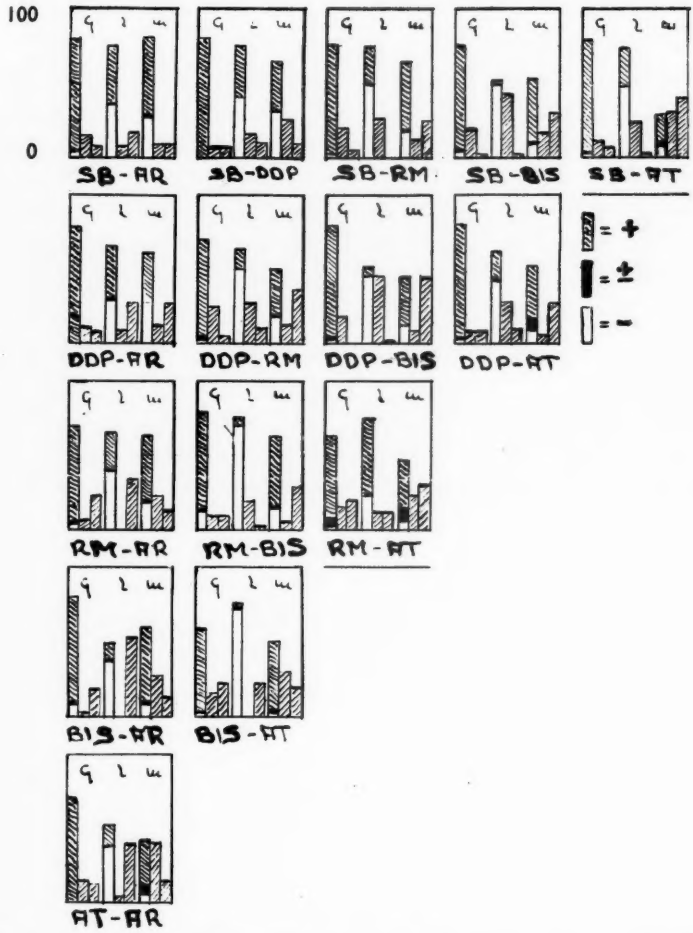


Fig. 1. — Comparison of the different techniques. (A more detailed explanation appears in the text.)

tion cannot be seen and sugar solution is subject to easy contamination. The incubation time, 6 hours, is longer than in the other two micromethods, and the results are not as strikingly distinct as in the others.

The third Clarke-Covan's enzymatic method in capillary tubes, included in the preliminary test series, is cumbersome in its technic. The filling of the capillary tubes and incubation is rather complicated and can hardly be used in routine laboratories. Also the

change of colour is difficult to observe, at least with the indicator concentration mentioned in the directions.

Figure 1 shows a comparison of the results obtained by the different methods. Each of the diagrams has three triple-columned sections, one for each sugar. The first column in each set gives the number of the concordant results as a percentage: its lowest part may be blank indicating the percentage of concordant negative results, its middle part black indicating the concordant doubtful results and its upper part with oblique shading indicates the concordant positive results; the second column gives the relative amount of positive results obtained only by the first-mentioned technic, and the third gives the percentage of the positive results obtained only with the latter technic. The first horizontal line of diagrams shows the results of the sugar broth tube tests compared with those of the other methods; the second those obtained with DDP compared with others, and so on.

On the whole the determinations made with anthrone reagent and sugar broth agree fairly well. The negative results obtained with anthrone reagent may be increased by an increase in reducing substances during the incubation e.g. if the disaccharides become hydrolysed quickly. Anthrone-positive results on the other hand are not overshadowed by an alkali formation which can occur in the sugar broth tubes and there cover a weak acid formation. The anthrone technic is inferior to the rapid technics with disaccharides and the sugar broth is less effective than the anthrone technic with regard to glucose-positivity when the strains are known to be strong alkali producers (cf. Table 1 Proteus and Pseudomonas). In order to assess the role of alkali formation e.g. in sugar broth it is thus obvious that sugarfree indicator broth tubes should be used simultaneously. Neither the SB nor the AR technic, accordingly, can be regarded as absolute indicators of the fermentation of sugar or as absolute control for fermentation methods.

For a comparison of the acid formation results obtained with the different technics it might be well to take the most sensitive technics as points of departure. Taking the maximum sensitivity as ideal the different technics can be grouped as follows:

TABLE 3

	Glucose	Lactose	Maltose
1.	SB 90% positive	AR 48% positive	BIS 74% positive with
2.	AR 90	SB 45	AT 65 31 strains
3.	DDP 87	DDP 35	RM 51
4.	AT 84	RM 23	SB 51
5.	BIS 77	AT 19	AR 48
6.	RM 68	BIS 3	DDP 42

If on the other hand sugar broth tubes are considered the standard the value of the various technics is as follows:

TABLE 4

Glucose	Lactose	Maltose
SB — 90% positive	SB — 45% positive	SB — 51% positive
AR ±0%	AR +6.6%	BIS +45.1%
DDP — 3.3	DDP —22.2	AT +27.4
AT — 6.6	RM —48.8	RM ± 0
BIS —14.4	AT —57.7	AR — 5.8
RM —24.4	BIS —93.3	DDP —17.6

The values obtained for the glucose fermentation by macro-technics (SB, AR and DDP), and even AT, agree well. Other micro-technics, however, give too few positive results.

Lactose, which is known to be a slowly-decomposed sugar, gives considerably fewer concordant results with the new technics and the enzymatic technics in particular give too many negative results. Owing to the slow acid formation too negative results occur, caused probably by an alkali formation, even when using the SB technic.

With maltose, sugar broth and anthrone (cf. AR results) give fairly concordant results; however, a probable decomposition of disaccharide can increase the negative results with anthrone. The new technics on the other hand give even more discordant results than they did with lactose. Significant is the frequent occurrence of positive variation in the enzymatic technics. Since the variations

are caused in the first place by Proteus and Pseudomonas strains, alkali formation in sugar broth and the decomposition of disaccharide in anthrone may be partly responsible for the difference.

CONCLUSION

Each of the technics has its own advantages. Many of them have considerable theoretical interest. It must be remembered, however, that our generally accepted picture of the sugar fermentation of bacteria is based on broth tests and that it continues in consequence to be the most serviceable in routine diagnostics. If we want to use rare sugars in routine diagnostics the DDP technic may prove serviceable but the results must be interpreted with caution.

As lactose fermentation is one of the most important criteria in the fermentative identification of intestinal bacteria, microtechnics, especially the enzymatic ones, do not appear suitable for routine diagnostics despite the rapidity with which results can be established. They provide us for continued investigations with too many bacterial strains fermenting lactose.

REFERENCES

1. Spaur, C. L., and Wynne, E. S.: Texas Rep. Biol. & Med. 1951:*9*:353.
2. Hannan, J., and Weaver, R. H.: J. Lab. Clin. Med. 1948:*33*:1338.
3. Clarce, P. H., and Cowan, S. T.: J. Gen. Microbiol. 1952:*6*:187.
4. Morris, D. L.: Science. 1948:*107*:254.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SOME EXPERIMENTS ON THE EFFECT OF AUREOMYCIN ON THE FERMENTATIVE POWER OF ESCHERICHIA COLI

by

R. KOULUMIES [1], KAINO J. AALTONEN and MATTI KANNISTO

(Received for publication February 26, 1953)

It has been demonstrated in numerous experiments (1, 2, 4, 5, 6, 7, 8) that the resistance of bacteria to antibiotic drugs is increased when the bacterium is grown under the continuous action of the drug. The degree of increased resistance is, of course, variable (1, 3, 4, 5, 8), but in most cases there is a manifold increase as compared with the original resistance.

An interesting question is whether this increase in the resistance also produces changes in other properties of the bacteria, such as their fermentative power. However, in experiments made by us on changes in the fermentative power of *Escherichia coli*, using 15 different carbohydrates, negative results were obtained in most cases after 24 hours (Table 1).

On the second, third, and fourth day the findings were the same except in the case of dextrin and salicin. This gave us reason to study the decomposition of these carbohydrates more closely.

Pure cultures of 20 strains of E. coli were isolated from the faeces on Drigalski-Conradi plate. The original strains were stored on slant cultures at room temperature.

The *p*H of the meat broth used for increasing the resistance of the strains was buffered to 7.3—7.4 (Beckman's *p*H-meter). The broth was distributed into bottles containing a sufficient amount for each day's use and was sterilized in the autoclave. Before use

[1] Aided by a grant from the Sigrid Jusélius Foundation.

TABLE 1

DECOMPOSING POWER OF E. COLI ON CARBOHYDRATES BEFORE AND AFTER TREATMENT WITH AUREOMYCIN

(Time of Observation 24 hrs.)

	Arabinose	Xylose	Rhamnose	Galactose	Glucose	Mannose	Lactose	Maltose	Sucrose	Trehalose	Raffinose	Dextrin	Mannitol	Inositol	Salicin
B = Before A = After	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.	B.A.
Strain I ..	++	++	++	++	++	++	++	++	−−	++	−−	++	++	−−	−−
Strain II ..	++	++	++	++	++	++	++	++	−−	++	−−	−−	++	−−	−−
Strain III..	++	++	++	++	++	++	++	++	−−	++	−−	+−	++	−−	−−
Strain IV	++	++	++	++	++	++	++	++	−−	++	−−	−−	++	−−	−−

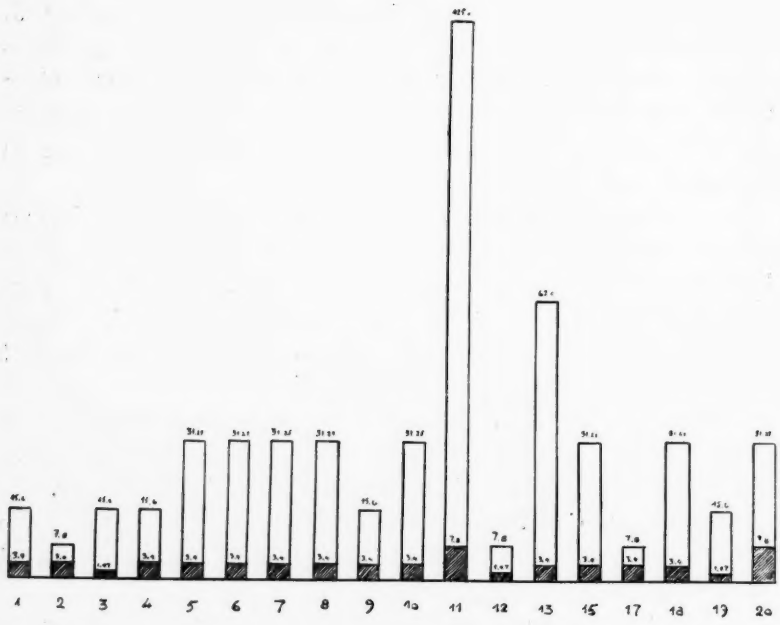


Fig. 1. — Sensitivity of 18 strains of E. coli. Shaded columns=strains grown in broth without aureomycin. White columns = strains grown in broth with aureomycin.

the *p*H was controlled. For the sensitivity measurements we prepared solutions of aureomycin hydrochloride (Lederle Laboratories) in broth in concentrations of 500, 250, 125, 62.5 and so on, down to 1.95 μg/ml. One milliliter of each dilution was pipetted for each strain.

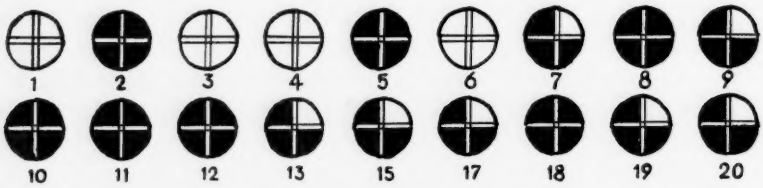
The plain broth was inoculated from plate cultures of the E. coli strains grown overnight and the broth was then incubated for 24 hours at 37°C. Dilutions of 10^{-4} were made from these cultures with sterile broth and 1 ml of the dilution was pipetted into the test tubes containing aureomycin. In this manner each of the 20 strains was grown in ten concentrations of aureomycin. After 24 hours the sensitivity of the bacteria to aureomycin was evaluated on the basis of turbidity as compared with the control tubes.

To increase the resistance to aureomycin the strains were grown for a period of 3 weeks in aureomycin broth by transferring them after about every 36 hours into new broth tubes containing rising concentrations of aureomycin. Final readings of the sensitivity were made at the end of the test period.

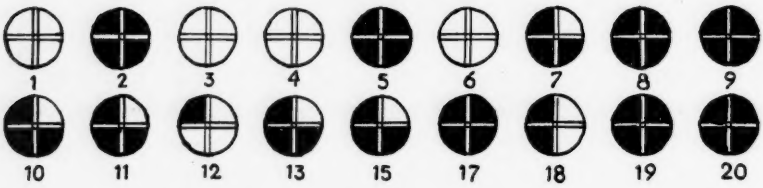
For control, cultures of the same strains were made in pure broth, with transfers also at 36-hours intervals. Final readings of the sensitivity were done after 3 weeks. When compared with the original strains, the resistance of the control cultures was found to be unaltered within the limits of error in the method employed.

The resistance values for the control cultures and the cultures grown in aureomycin broth, as well as the increase in resistance are shown in Fig. 1. Two strains (Nos. 14 and 16) were lost in the course of the experiment.

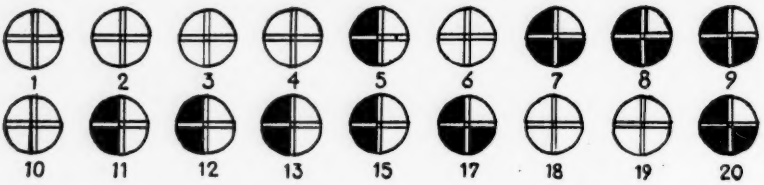
For comparison of the fermentative capacity of the strains grown in pure broth and those treated with aureomycin, cultures of each strain were made in broth containing 1 per cent solutions of dextrin and salicin. On fermentation of these carbohydrates by the bacteria the originally slightly alkaline broth will give an acid reaction because of the acid produced. Bromthymol blue was used as the indicator and the observations were made at intervals of 24 hours during 4 days. No change was observed in dextrin fermentation when comparing the treated and nontreated strains, whereas salicin fermentation had underwent a definite change, the course of which is seen from Fig. 2.



A. Original strains.



B. Strains after 3 weeks' culture in broth without addition of aureomycin.



C. Strains after 3 week's culture in broth with aureomycin.

Fig. 2. — Decomposition of salicin in broth by E. coli. Sectors show daily readings on 4 days. Black = positive reaction. White = negative reaction. Example: Strain A. 7 = 1 st day, negative; 2nd, 3rd and 4th days, positive.

SUMMARY

1. Resistance and carbohydrate metabolism were studied with 18 strains of *Escherichia coli* grown for 3 weeks in broth which contained increasing concentrations of aureomycin by comparing them with adequate controls.

2. At the end of 3 weeks the resistance of the E. coli strains to aureomycin had increased to 2—32-fold.

3. The increased resistance to aureomycin was not found to change the capacity of E. coli to ferment dextrin.

4. On the other hand, the experiments seemed to indicate that the capacity of E. coli to ferment salicin was retarded in the strains treated with aureomycin as compared with the controls. During

the first 24 hours no acid had been produced by any of the aureomycin-treated strains, whereas this had occurred with 7 of the 18 strains in both control groups.

REFERENCES

1. Finland, M., Collins, H. S., and Paine, T. F.: J. Amer. Med. Ass. 1948:*138:*947.
2. Fusillo, M. H., and Romansky, M. J.: Antib. & Chemoth. 1951:*1:*107.
3. Ge:on, H. M., and Fasan, D. M.: Science 1951:*114:*422.
4. Gocke, T. M., and Finland, M.: J. Lab. & Clin. Med. 1951:*38:*719.
5. Kaipainen, W. J.: Ann. Med. Exp. & Biol. Fenn. 1951:*29:*Suppl. 1.
6. Levinson, D. C., Griffith, G. C., and Pearson, H. E.: J. Amer. Med. Ass. 1951:*145:*261.
7. Marshall, H., Palmer, W., and Kirsner, J.: 1950:*144:*900.
8. Monnier, J. J., and Schoenbach, E. B.: Antib. & Chemoth. 1951:*1:*472.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SERUM LIPIDS IN THE INFRA-RED REGION

A SPECTRO-PHOTOMETRIC STUDY OF DIABETICS AND NORMALS

by

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(Received for publication February 26, 1953)

The serum lipids were extracted with boiling ethyl-alcohol, using one part of citrate plasma to 10 parts of alcohol. (The total plasma from 9 cc of blood was analysed.) After centrifugation, the supernatant was evaporated in the exsiccator above P_2O_5, dissolved in 5 cc of CCl_4, evaporated once again, and finally resolved in 0.5 cc of CCl_4. This solution was examined using a Beckman spectro-photometer, model IR2.

The absorption due to lipids was calculated by comparing the registered curve with that of CCl_4 and expressed in per cents. The measurements were performed after every 5.0 mm of the run, i.e., roughly after every 0.05—0.1 μ.

The first graphs in Fig. 1 gives us the mean value of four normal plasma absorption curves. It also shows the absorption curves of 5 per cent solutions of commercial egg lecithin, stearin, cholesterol, and sphingomyelin.

The general shape of the absorption curve of serum lipids resembles those of lecithin and tristearin-glycerid. It differs from the latter at many points, resembling somewhat the lecithin curve *f.i* between 8.87—10.34 μ. The cholesterol and sphingomyelin curves have a different character.

In order to study the possible differences between the lipids of

80 % — normal serum lipids
80 %

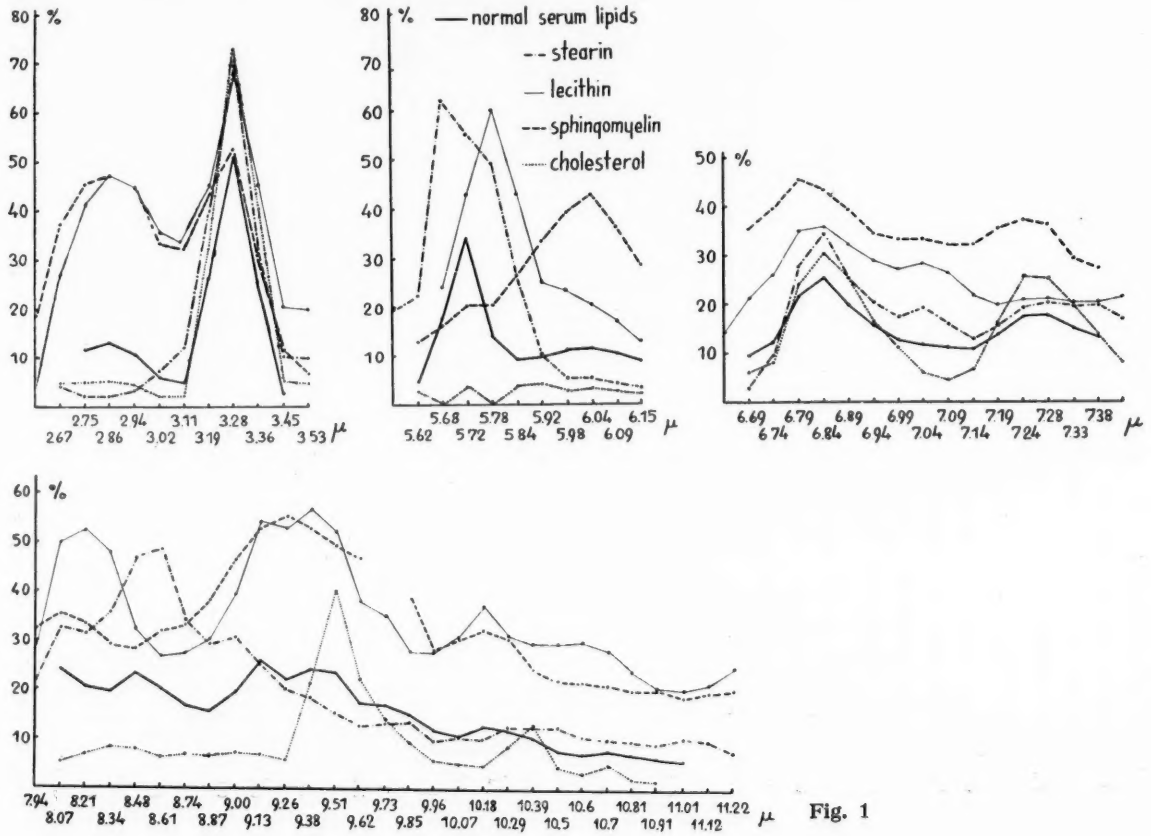

%
normal serum lipids
stearin
lecithin
sphingomyelin
cholesterol
μ

Fig. 1

normal and diabetic persons, we calculated the relative concentration sat each observed wave-length according to Beer's law: Conc. = —log (1-absorptance). We assumed that the absorption at each wave-length should be due to the same chemical compound. This approximation seems justified because the serum lipids are probably rather similar in composition.

Four normal young adults, nine young severely diabetic adults, and eleven over 50-year-old diabetics were examined. The results are collected in the graphs of Fig. 2. There are given the mean of the absorption percentages for the normals and a curve for the young and another for the elderly diabetics. The two last-mentioned curves were obtained by measuring at each wave-length the mean concentration of lipids of diabetics against the respective mean concentration of normal lipids and by converting the normal concentrations to 100%, *i.e.* to the 0% line.

When studying the mean aberration curves it is apparent that the two curves reflecting the lipids of diabetics do not greatly aberrate from the straight line of the normal lipids. In general, the oscillations donot exceed the +30% and —15% lines. When there is a peak in the absorption curve of normal lipids the aberration curves mostly approach the 100% line, and where the absorption of normal lipids is weak there is a peak in the aberration curves. In other words, where the lipids give strong absorptions the similarity of the absorptions overshadows the possible minor differences, which, on the other hand, dominate the picture if the normal absorptions are weak.

The analysis of the differences at most points is impossible because of the complexity of the possible explanations and because of a lack of knowledge on many essential points. Some simple deductions may, nevertheless, be made.

Considering the CH absorptions at 3.28, 6,84 and 7.28 μ we obtain for the lipids of young diabetics the values of 8, 4 and 9 per cent, respectively, above those of the normals. In other words, the CH skeleton of the lipids of young diabetics seems to exceed with some 7 per cent that of the normals. Correspondingly the CH values for the lipids of old diabetics are 95.95 and 99.5 per cent, i.e., averaging roughly 3.5 per cent below the normal values.

Both aberration curves for the diabetics show relatively strong absorptions between 2.94 and 3.11 μ, thus reflecting higher con-

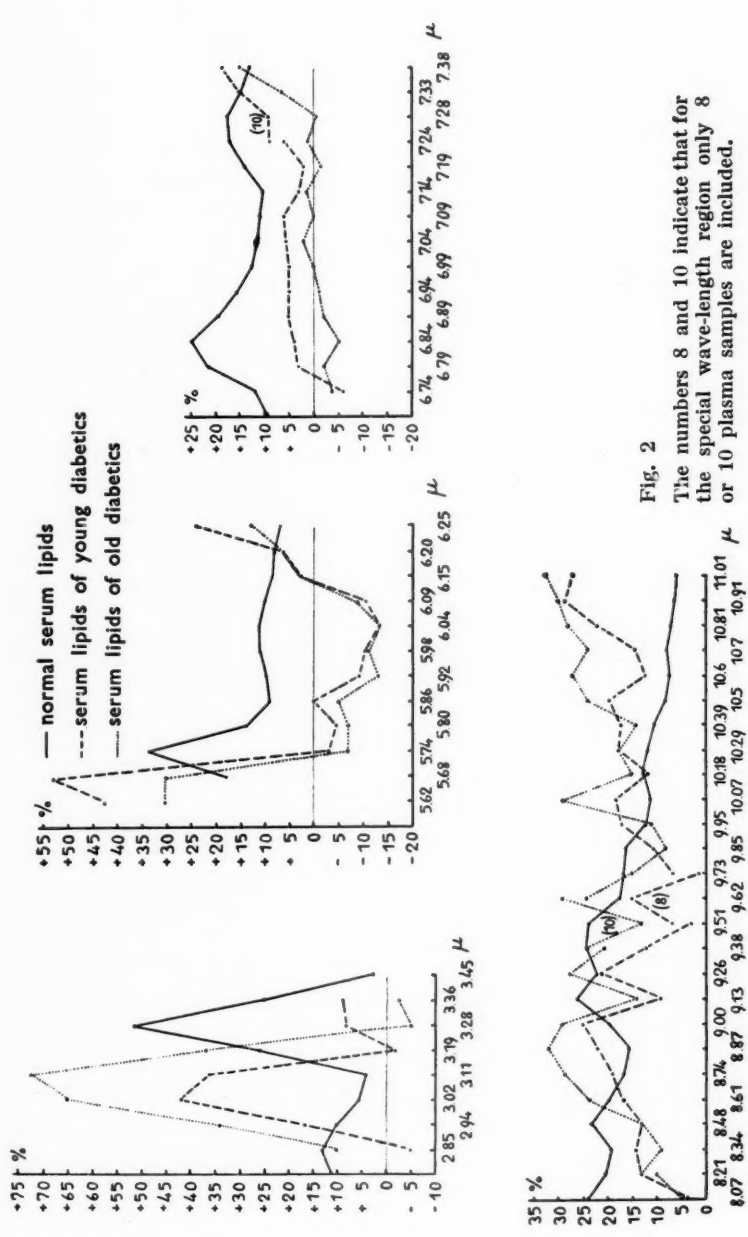


Fig. 2

The numbers 8 and 10 indicate that for the special wave-length region only 8 or 10 plasma samples are included.

centrations of the OH and NH groups than do the normal lipids. Taking, further, into consideration the fact that according to the CH absorptions the lipid values of old diabetics should lie below the normal line, there seems to be but little doubt that the NH value is relatively higher in the lipids of diabetics — of old diabetics at any rate. It may also be pointed out that the lipid aberration curve of old diabetics almost invariably lies highest throughout the region of 8.48 to 11.01 μ, where also the lecithin curve distinctly dominates over the triglycerid absorptions (Fig. 1). This part includes also the phosphate absorptions. Accordingly there are reasons to assume that the lipids at least of old diabetics contain more N- and perhaps P-lipids than the normal ones.

Another characteristic difference between the lipids of normals and diabetics is to be found between 5.74 and 6.09. Here the lipids of diabetics, and especially those of young diabetics, show rather weak absorptions. At this wave-length the absorptions are generally due to amido compounds and double bonds (1). Thus sphingomyelin (Fig. 1) is strongly absorbent here.

The lipids of young and old diabetics differ certainly on many other points too, which is reflected throughout the aberration curves, but, as stated above, we are not ripe at this stage to offer explanations. The lipids should be chemically split and fractioned before attacking this problem.

SUMMARY

The infra-red absorption curves of plasma lipids of four normal individuals, nine young and eleven old diabetics, were studied in CCl_4. The relative concentrations were calculated from the net absorption curves according to Beer's law, and the mean concentrations were calculated from the individual relative concentration curves. On the basis of these calculations the lipids of diabetics were measured against the lipids of normals, and aberration curves were drawn.

When analysing the aberration curves it becomes evident that the mean curves show similarities and differences. The lipids of diabetics and especially those of old diabetics seem to contain, on the average, more N-, P-lipids than those of the normals and

especially those of young diabetics less amide groups and perhaps double bonds.

There are, indeed, great variations within every group, and the differences between the normal and diabetic lipids overlap. We are also aware that the differences we have seen may have been due not only to the disease but to the dietary differences between the groups even if the blood samples compared are taken in the morning.

REFERENCES

COLTHUP, N. B.: Spectra-Structure Correlation in the Infra-Red Region. J. opt. soc. Amer. 40. 307. 1950.

FROM THE PHYSIOLOGICAL DEPARTMENT OF THE ZOOLOGICAL INSTITUTE AND THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

DEVELOPMENT OF VISUAL CELLS AND PHOTOMECHANICAL MOVEMENTS IN AMPHIBIA [1]

by

LAURI SAXÉN

(Received for publication March 3, 1953)

Amphibia constitute an interesting subject for the study of sensory physiology since they are vertebrates which in their embryonal stage are independent, active individuals. The development of the eye, and particularly of the visual cells, in amphibia has been extensively investigated. The function and functional development of the visual cells during the metamorphosis has been devoted much less attention.

The most natural and generally-used method of estimating the vision of tadpoles is to observe their spontaneous reactions to light. However the unreliability of this method has become evident since several researchers (9, 3, 12) have found the photokinesis of the tadpole to be due in the first place, to cutaneous receptors and to be independent of the eye. This potential error is eliminated in the optomotoric method developed by Schlieper (13), and the experiments effected with it (2) have yielded excellent results with amphibians too. In the youngest tadpoles of Rana temporaria studied (the equivalent of the 10-day old tadpoles in the present material) the cones function and subsequently remain unchanged. The function of the rods is almost non-existant at this stage,

[1] Preliminary report.

gradually gains force and becomes fully effective only after the metamorphosis. Similarly, Wald (14) has found that the absorption maximum of the photosensitive substances of Rana catesbyana move during the development towards the short-wave end of the spectrum.

The photomechanical movements of amphibians in the larval stages have attracted fairly little attention. Detwiler (5) has studied the larval Ambystoma in a stage when both the rods and the cones still had a typical vacuole and, hence, cell differentiation was only just beginning. He was able to observe distinct cone movements and pigment migration, while the rods were immobile. Laurens and Williams (10), in adult individuals of the same species, found exactly the same conditions; the extent of pigment migration was even less. Birukow (2) considers it probable that with even the youngest Rana temporaria tadpoles mentioned the cone myoid is contractile. According to Majima's (11) investigations the cones of week-old tadpoles (Bufo vulgaris) react slightly to light. Pigment migration is first found in them considerably later only. The photomechanical movements of Xenopus laevis have not been studied before, and the only mention of the adaptation of this species is by Hess (8), according to whom it is in a class with Bufo and Triton.

MATERIAL AND METHODS

The investigations were effected with two anuran species: Rana temporaria L. and Xenopus laevis Daudin. The material consists, in addition to adult animals, of a simple development series of 1—56 days, with 12 stages of development for each species. As the preliminary and final investigations were made with separate, independent development series, two materials were available for possible control studies. Each final adaptation test involved 5+5 animals, and the measurements were taken from 3 + 3 preparations, the technically most successful ones of the 1 st lot.

The experiments were carried out as follows: dark adaptation 24 hours, fixation in weak red light followed by another 24 hours in darkness to prevent possible post-mortal movements. Simultaneously, the animals to be adapted to light were kept for 24 hours in the corresponding fluid volume during the last 6 hours they were exposed to indirect daylight, 500 Lux. Fixation was effected

with neutral formol-alcohol, and always at the same time of the day in order to eliminate any error from possible diurnal rhythms (1). 7 μ serial sections in frontal plane were made, and stained with aniline blue-orange-azocarmine (Heidenhain) or molybdic acid haematoxylin-eosin (Held).

The photomechanical movements were measured to the nearest millimeter from a magnification 2000 $\times$ drawn with Abbe's drawing apparatus; the results are the mean values of 3 $\times$ 30 measurements. The result has been indicated in terms of distance: (a) m. lim. ext. — oil droplet of the cones, (b) m. lim. externa — the borderline between the outer and inner segment of the rod, (c) m. lim. ext. — the nearest pigment particle (Rana) or total width of pigment cell layer (Xenopus). Cells per field of vision (diameter 120 μ) have been counted from 3 $\times$ 10 fields of vision. All measurements wree taken with an immersion lens from the fundus, from sections in which the plane of section is parallel to the plane determined by the outer segments of the rods.

RESULTS

Histology. — Four types of visual cells are distinguished in the retina of adult Rana temporaria: red rod, green rod, single cone and double cone. As the first two types cannot be distinguished from one another in the larval stages, the rods are taken in the following as a single group. Per field of vision, the cells average 17.9—14.6—3.6, equalling a total density of 3.0 $\times$ 10^4 sq.mm. The retina of Xenopus laevis has three types of visual cells: single (red) rod, cone I and cone II. The cones differ in size — all the former's dimensions are approximately twice those of the latter — as well as in the fact that type II is immobile and fails to exhibit any positional changes to light. For the same reason as above the two cone types are reckoned as one group in density calculations. The green rods previously encountered in Xenopus (4) could not be discerned with the technique employed. The relative frequency of the cells is 14.8 and 13.9, and the total frequency 2.4 $\times$ 10^4 per sq.mm.

Development. — The differentation of the visual cells of Rana temporaria begins 4—5 days after hatching, when the first rods and cones are discernible in the fundus. No double cones or their possible progenitors are found. At this stage all the cells still show

a typical achromatic vacuole, which disappears from the rods at an age of 7—10 days. The first double cones are discernible 7 days after hatching. After this neither of the two cone forms undergoes any considerable change, whereas the outer segments of the rods double their length. Visual cell density increases very nearly rectilinearly during development. The rod/cone ratio remains unchanged (1.3), while the percentage of double cones decreases considerably (18.6—10.0), and the total of visual cells rises 2.7 times (13.4—36.1).

Cell differentiation sets in somewhat earlier with Xenopus laevis than with the previous species, at the age of 2—3 days. The next stage of development (5 days) sees the appearance of the first rods with no vacuoles, at the same time a number of the cones can be discerned as belonging to type I or II. Later the growth of the outer segment of the rod is most prominent; the elongation is 5-fold. The cone types remain unchanged during development. The most prominent difference compared with the development of Rana temporaria is that the visual cell density remains the same throughout development (29.0—28.7). The rod/cone ratio is also unchanged (1.1).

The above results prompt the formulation of certain physiological conclusions. Photopic (cone) vision develops with both species at a relatively early stage. With Rana it evidently continues to develop as the cone density increases; with Xenopus it remains unchanged. Therelative cone density of adult Xenopus is somewhat higher and its photopic vision, evaluated on this basis alone, is accordingly more developed. Scotopic (rod) vision develops later than the former and evidently at a slower rate. The considerably higher rod density of the Rana seems to indicate that its sensitivity to light exceeds that of Xenopus.

Photomechanical Movements. — Rana temporaria: the photomechanical movements of the adult animal have been much investigated. The results show conclusively that the species has fairly well-developed cone and rod movements and pigment migration. The adaptation tests reported on above led to the same conclusions. The extent of cone and rod movements and pigment migration, with adult animals, was 13.7 μ, 9.6 μ and 16.1 μ, respectively. Their percentages of the total thickness of the visual cell layer are 23, 14 and 27.

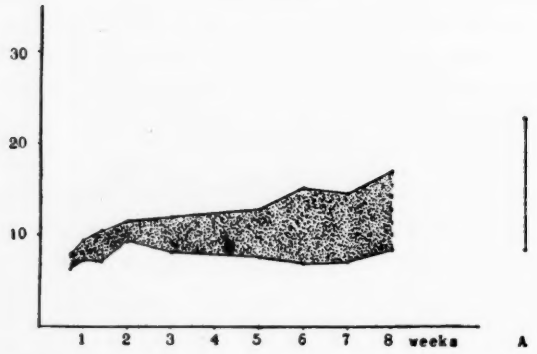


Fig. 1. — Diagram showing cone movements of Rana temporaria in the various stages of ontogeny.

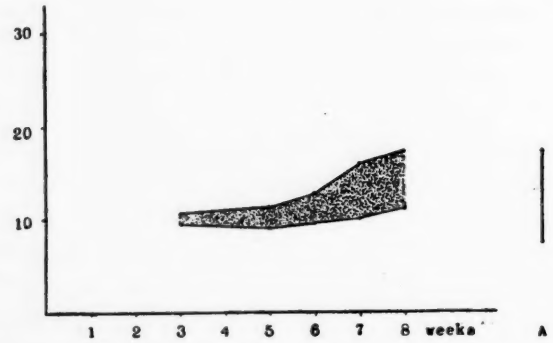


Fig. 2. — Diagram showing rod movements of Rana temporaria in the various stages of ontogeny.

Development (Figs. 1—3). — In the stage where the rods and cones are first histologically distinguishable from one another (5 days), no distinct movements can be observed. An occasional individual cone is found to have elongated, but the results of a more comprehensive measurement show that the differences lie within the error margin. The rods and pigment are immobile, both in a fixed dark position. The cone movements continue to develop: first more slowly, but towards the end of the metamorphosis at a greater rate — with the oldest tadpoles they exceed half (8.4) the corresponding movement of the adult animal. The rods remain immobile up to the age of 2—3 weeks, but their subsequent development is fairly rapid and by the last stage of development they

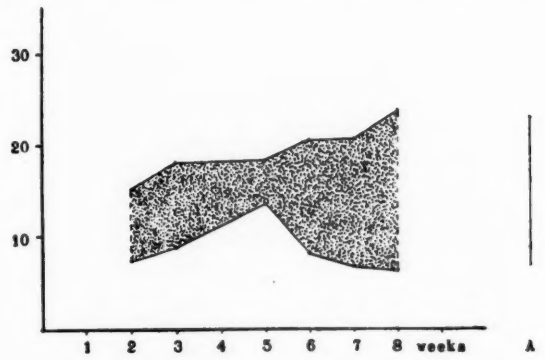


Fig. 3. — Diagram showing pigment migration of Rana temporaria in the various stages of ontogeny.

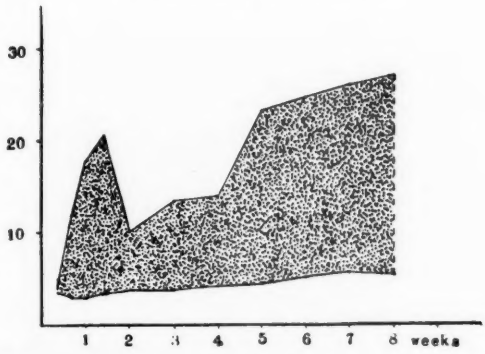


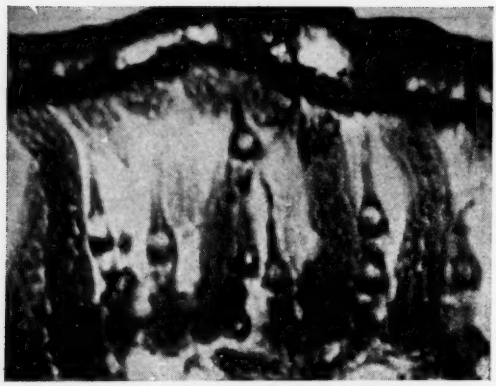
Fig. 4. — Diagram showing cone movements of Xenopus laevis in the various stages of ontogeny.

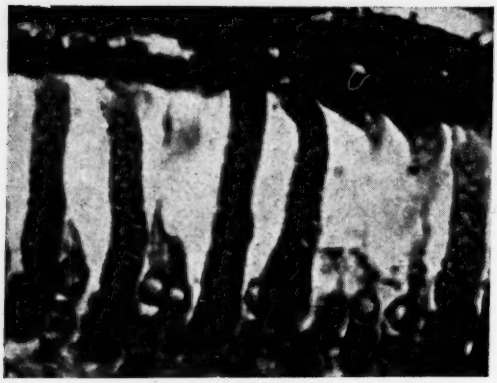
attain a movement some two-thirds of that of the adult animal. Pigment migration begins at 2 weeks, is immediately relatively extensive (7.7) and attains its maximum by the last larval stage.

Xenopus laevis. The table below gives a clear picture of the photomechanical movements of the adult individual.

TABLE

	Dark Adapted	Light Adapted	Difference
Cones	42.3	10.7	+31.6
Rods	11.8	14.2	+ 2.4
Pigment	25.0	24.7	— 0.3





Figs. 5 and 6. — Photomicrographs of the dark-adapted and light-adapted retina of a 35-day old Xenopus laevis tadpole. Heidenhain's azane. × 1000.

The results show that the cone movements are highly developed (51%), the rods move very slightly (4%) and the pigment is completely immobile. This kind of picture is typical of many urodeles (15, 6), and the eye of the Xenopus may be considered, primarily, as the intermediate form between anuran and urodelous amphibians.

Development (Fig. 4). — The cone movements begin at a very early stage; at the age of 5 days the extent of movement is already 8.8 μ. The movements continue to develop vigorously, reaching their first peak with 10-day old tadpoles, but decline subsequently.

This sudden weakening of movements, distinctly ascertainable in both of the materials studied, persists for the next three weeks of development. In the external development of the tadpole this date coincides with the stage preceding the appearance of the extremities. In the 5th week the movements regain their former extent, develop further and by the end of the metamorphosis equal approximately two-thirds of the movements of the adult animal. No rod movements or pigment migration can be observed during the development.

CONCLUSIONS

Prior to setting down the conclusions reached it must be pointed out that they are based on a single phenomenon, important though it is in the vision of the lower vertebrates. Clearly, therefore, we are not entitled to estimate the development of vision on this basis alone. On the other hand we can assume that these light reactions and their comparison provide certain indications for evaluating the development and nature of the vision of the different stages of ontogeny.

The youngest, 1—4-day old, tadpoles of Rana temporaria are evidently incapable of seeing. Photopic vision then develops slowly, and scotopic vision only considerable later. In both the development continues after the metamorphosis. However, it is incorrect to estimate the development of photopic vision only by the reactions of the participating elements, i.e. the cones. According to the classical explanation advanced by Garten (7), it is expressly the rod movements and pigment migration that are very important to great activity of vision, and hence we can take it that the development curve of the photopic vision of the tadpoles studied rises considerably more steeply towards the end of the metamorphosis and with the movements in question developing than the cone movements alone indicate. By contrast, we cannot assume that the early start of cone movements indicates that the scotopic vision is advanced at this early stage and results, in accordance with Garten's theory, in unobstructed rodfunction. It is more likely that a condition for movements is brought into being by the earlier structural development of the cones, and that they occur in spite of the fact that, from the point of view of the still un-

developed rod mechanism, they would not be necessary. The results obtained, therefore, are in keeping with Birukow's results reported above.

A similar development can be observed in the eye of Xenopus laevis also, although the adaptation mechanism is simpler. The extensive cone movements of the younger larval stages do not, in this case either, indicate the existence of scotopic vision but only the development and reactivity of the cones. With the rods gradually developing into functionary visual cells the cone movements decline, and the result is a typical primitive adaptation eye in which both of the mechanisms function in accordance with the duplicity theory. As development progresses the cone movements grow and towards the end of the metamorphosis, in contrast to Rana, mainly scotopic vision develops. Only after the metamorphosis do weak rod reactions set in, with a resulting increased acuity of vision. The end result, however, is an eye which must be considered primarily an organ of scotopic vision, in spite of the fact that the relatively high cone density partially compensates for the rod movements and pigment migration required for high activity of vision.

REFERENCES

1. Arey, L. B., and Mundt, G. H.: Anat. Rec. 1941:*79:*5.
2. Birukow, C.: Z. vergl. Physiol. 1949:*31:*322.
3. Cole, W. H., and Dean, C. F.: J. Exp. Zool. 1917:*23:*361.
4. Denton, E. J., and Pirenne, M. H.: J. Physiol. 1951:*115:*33 P.
5. Detwiler, S. R.: J. Comp. Neur. 1923:*36:*113.
6. Detwiler, S. R.: Vertebrate Photoreceptors. New York, 1943.
7. Garten, S.: Graefe-Saemisch Handb. d. Augenheilk. 1907:*3:*250.
8. Hess, C.: Pflügers Arch. 1910:*132:*255.
9. Laurens, H.: J. Exp. Zool. 1914:*16:*195.
10. Laurens, H., and Williams, J. W.: J. Exp. Zool. 1917:*23:*71.
11. Majima, K.: Graefes Arch. 1925:*115:*286.
12. Obreshkove, V.: J. Exp. Zool. 1921:*34:*236.
13. Schlieper, C.: Z. vergl. Physiol. 1927:*6:*453.
14. Wald, G.: Harvey Lect. 1946:Ser.XLI:117.
15. Walls, G. L.: The Vertebrate Eye. Bloomfield Hills, 1942.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY AND THE CHILDRENS' CLINIC, UNIVERSITY OF HELSINKI

ETIOLOGY OF INTERSTITIAL PLASMA CELL PNEUMONIA

by

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(Received for publication March 13, 1953)

The clinical features of interstitial plasma cell pneumonia have been described earlier (1, 2) and are known in several countries, but the etiology of this condition has remained obscure. Some authorities consider it to be an infectious disease caused by a virus. Though investigations aiming at the isolation of the virus have been carried out, its existence has not as yet been conclusively demonstrated. In the Virus Laboratory of the University Department of Serology and Bacteriology, Helsinki, investigations have also been carried out on patients from the Childrens' Clinic and the Childrens' Castle (Helsinki). Although the results have been practically negative, we feel justified in reporting them.

MATERIAL AND METHODS

In order to infect embryonated hens' eggs and the experimental animals the following materials were used: lung tissue from nine infants who had died of interstitial plasma cell pneumonia and blood from two patients. The throat swabs and the blood samples were obtained from fresh untreated cases. The diagnosis of the post mortem cases was verified by a histological examination of the lungs. The diagnosis in the two non-fatal cases was based on typical clinical features and roentgenological findings. The autopsies were performed 9 to 33 hours after the death of the patient. Between death and the autopsy, the bodies were kept at a temperature of + 4 °C. In some cases it was, for practical reasons, not possible to perform the inoculation immediately

after the autopsy; in these cases the samples were kept deep-frozen (at about — 20 °C.). The piece of lung tissue was ground with quarz sand and from it a 10 or 20 per cent suspension in 0.3% buffered bovine albumin was prepared. This was then centrifuged for 20 minutes at 1500 r.p.m. For the experiment, embryonated hens' eggs, white mice, young guinea pigs and kittens were used. The eggs were inoculated either in the amniotic cavity or the yolk sac. In the former cases, eggs incubated for 7 to 12 days at + 37.5 to + 38 °C were used. After the injection. of 0.1 to 0.2 cc of the lung suspension, the eggs were kept at + 36 °C. From two to nine (on an average, five) passages were performed; the duration varying from three to ten days. Haemagglutination tests were performed on the amnion fluids using a 0.5% suspension of hen's blood cells as an indicator. For the inoculation of the yolk sac, eggs incubated for 6 or 7 days were used and 0.5 cc of the lung suspension was injected. Two to five passages were performed, the duration varying from four to nine days. From every yolk sac, a preparation was made on a slide and stained by Macchiavello's method. Only in exceptional cases were penicillin (1000 units per cc) and streptomycin (1250 units per cc) used. In the animal experiments, white mice aged from one to seven days, young guinea pigs weighing between 100 and 150 g, and kittens aged from five to twenty-one days were used. The animals were infected intranasally or intraperitoneally with ether narcosis and, when blood specimens were used, also intramuscularly and subcutaneously. The specimens used for the infecting of the animals and the number of the specimens are presented in table 1 and the number and duration of the passages in table 2.

TABLE 1

	Lung Samples	Throat Swabs	Blood Samples	Amnion Fluid
mice	4	1		1
guinea pigs	1		2	
kittens	2	1		

TABLE 2

	Number of Passages	Duration
mice	2—3	4—35 days
guinea pigs	1—2	20—23 days
kittens	1—3	8—31 days

A histological examination was performed on every animal passage. One guinea pig and one series of mice were treated with cortisone (Cortone, Merck), one mg per 100 g of body weight, as there are reports in the literature indicating that this drug makes the experimental animals more susceptible to certain viral infections (4). In order to prevent bacterial infections, penicillin (1000 μ/cc) and streptomycin (2500 μ/cc) were administered in most of these cases.

RESULTS

1 269 embryonated eggs were used for the investigation of five different lung samples. Excluding the chicken embryos which died within 72 hours after the inoculation (these deaths evidently being due to technical accidents), twenty-five embryos, i.e. 12%, died between 3 to 10 days after the inoculation, while no bacterial infection could be detected in the sterility controls. In some amniotic fluids, a positive haemagglutination test was found, but it was negative in the next passages; evidently this reaction was not due to a virus. In the yolk sac preparations, stained after Macchiavello, nothing pathological could be found.

2. One hundred white mice were used for the investigation of four lung samples, one throat swab, and amniotic fluid from one egg. Four mice died 4 to 15 days after the inoculation. In one of these, histologically, a bronchitis was found; in the lungs of one there were small areas of interstitial inflammation and in two a granulocytic pneumonia. In the other mice, a histological examination of the lungs revealed nothing pathological.

3. Five guinea pigs were used for the investigation of one lung sample and two blood samples. The results were negative.

4. Thirteen kittens were used for the investigation of one fresh and one deep-frozen lung sample (several months old), as well as one throat swab. The fresh lung specimen was inoculated into a kitten five hours after the autopsy, which was performed fifteen hours after the death of the patient. In the first passage, a histologically mild interstitial pneumonia was found; in the second, a disseminated and in the third, a well developed one. Because of a technical accident the investigation with this sample was discontinued. Of the three kittens infected with the preserved sample, in one, histologically, only slight interstitial cellular infiltrations

were found. In the kittens infected with the throat swabs, no pathological changes of the lungs were found. None of the kitten exhibited clinical signs of sickness.

SUMMARY

Lung specimens, throat swabs, and blood samples from interstitial plasma cell pneumonia patients were inoculated into eggs and different experimental animals. The results were negative, with the exception of a fresh lung sample, where in three passages a histologically mild interstitial pneumonia was found.

REFERENCES

1. AHVENAINEN, E. K., SOMERSALO, O., and YLINEN, ANNELI: Ann. Med. Int. Fenn. 1950:*39*:222.
2. WEISSE, KARLA: Z. Kinderhk. 1949:*67*:54.
   WEISSE, KARLA: Erg. inn. Med. Neue Folge. 1951:*2*:610.
3. HALLAUER, C.: Schweiz. med. Wschr. 1952:*82*:409. (extensive bibliography)
4. SCHWARTZMAN, G., and FISHER, A.: J. Exp. Med. 1952:*95*:347.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SENSITIVITY OF PSEUDOMONAS AERUGINOSA TO ANTIOBIOTICS

by

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Pseudomonas infection is often difficult to control with the common antibiotics. Its somewhat increased incidence in recent years in the routine samples has stimulated us to study the sensitivity of our strains to the newest antibiotics.

TABLE 1

INCIDENCE OF THE PSEUDOMONAS STAINS ISOLATED BY THE DEPARTMENT, HALF-YEARLY

Time	Total of Samples	Pseudomonas							
		Throat	Expect-oration	Blood	Feces	Urine	Pus	Total	%
1949									
1.7—31.12	2763		1	6		14	13	34	1.2
1950									
1.1—30.6	2979			1		23	16	40	1.3
1.7—31.12	3089			1		31	14	46	1.5
1951									
1.1—30.6	3130					38	42	80	2.5
1.7—31.12	3267	1		1	2	37	19	60	1.8
1952									
1.1—30.6	4446		1	1	3	42	30	77	1.7
1.7—31.12	3894		4		4	28	46	82	2.1

MATERIAL AND METHODS

The sensitivity determinations were made with 60 different Pseudomonas strains isolated in 1952. Thirtyfour of them had been isolated from pus samples, 22 from urine, 2 from feces and 2 from expectorations. With practically all the strains a preliminary routine determination was performed of sensitivity to sulfathiazole, penicillin, streptomycin, aureomycin, chloromycetin and terramycin.

Our investigation covers the determination of the sensitivity of the strains to polymyxin B, bacitracin, neomycin, terramycin and gantricin (3.4-dimethyl-5-sulfanilamido-isoxazole).[1]

The general sensitivity limits had to be defined first. For this purpose a tube dilution series in broth was made of each antibiotic and gantricin; this broth was inoculated with 4 arbitrarily selected Pseudomonas strains, 0.5 ml of an 8-hour serum broth culture, dilution 10^{-2}. The broth containing antibiotics was incubated for 20 hours at 37° C.

TABLE 2

INHIBITION OF GROWTH

Strain	Polymyxin B γ/Ml			Bacitracin units/ml			Neomycin mg/ml			Terramycin mg/ml			Gantricin mg/ml		
	50	5	0.5	375	187	93	1	0.1	0.01	1	0.1	0.01	0.8	0.08	0.008
48	—	—	+	—	—	+	—	—	+	+	+	+	—	—?	+
59	—	—	+	×	×	×	—	—	+	—	—	+	—	—	+
60	—	—	+	—	+	+	—	—	+	×	×	×	—	—	+
62	—	—	+	—	—	+	—	—	+	—	—	+	—	—?	±

\+ = growth as in controls
± = growth not quite as good as in controls
—? = growth poor
— = no visible growth
× = not made

From the mean inhibitory concentrations obtained a 100-fold concentration was selected and the paper discs (dia. 15 mm) were immersed in it. The moist discs were placed on inoculated plates. The concentrations of the solutions of antibiotics used were: polymyxin B 500 γ/ml, bacitracin 11,500 units/ml (a higher concentration could not be reached), neomycin 10 mg/ml, terramycin 10 mg/ml and gantricin 8 mg/ml.

[1] Polymyxin B and Bacitracin was received from Messrs. Havulinna Oy, the chief agents of Chas Pfizer & Co, Neomycin from Lääke Oy and Gantricin from Hoffman-La Roche & Co. I wish to express my best thanks to all the firms.

The disc test was carried out in Petri dishes, diameter 15 cm, so that all the determinations for each strain could be made in one and the same dish. The culture medium for the test consisted of blood agar of the following composition:

beef extract	0.3
NaCl	0.5
glucose	1.0
agar	2.0
water	1000.0 pH 7.4

An addition of 10% of normal cattle or horse blood was made.

A loopful of each strain from a 20-hour serum broth culture was inoculated into a 2 cc serum broth tube, incubated for 4 hours, and diluted to 10^{-1} with sterile saline solution. The test plate was inoculated with a cotton wool plug dipped into this mixture. Readings were taken after an incubation time of 20 hours.

RESULTS AND DISCUSSION

The results obtained in the disc test are given in Table 3.

TABLE 3

DISTRIBUTION OF STRAINS BY DIAMETERS OF INHIBITION ZONES

Antibiotic	Concentration	resistant → sensitive				
		—	—?	±	+	++
Polymyxin B	500 γ/ml	9	6	19	26	—
Bacitracin.....	11,500 units/ml	6	7	35	10	2
Neomycin	10 mg/ml	—	—	6	46	8
Terramycin	10 mg/ml	34	14	11	1	—
Gantricin	8 mg/ml	39	4	17	—	—

— = completely resistant
—? = inhibits growth to some extent
± = inhibition zone 1—2 mm or partially wider
\+ = inhibition zone complete 2—10 mm
++ = inhibition zone complete over 10 mm.

At the concentration employed, neomycin alone proved effective against all the strains, with an inhibition zone of over 2 mm in diametre. The second place is held by bacitracin and polymyxin B. Gantricin did not achieve complete inhibition with any of the

strains. The details are reported in the following when the different antibiotics are discussed.

Polymyxin B. — Polymyxin is generally considered the appropriate therapeutic agent in Pseudomonas infections. However, its toxicity restricts its use. According to literature, the Pseudomonas strains are sensitive to concentrations of 2 γ/ml (2) or 0.64—3 γ/ml (1) in broth cultures. In the present investigations, however, where the concentration 500 γ/ml probably equals a broth culture concentration of 5 γ/ml, 15 per cent of the strains were completely resistant to polymyxin, 10 per cent were fairly sensitive, and 43 per cent only could be considered sensitive.

As the inhibition zones with polymyxin seemed surprisingly small, another 10 bacterial strains were examined; they included strains assumed to be sensitive to polymyxin (E. coli and s. paratyphi B). The results are given in Table 4.

With these strains, too, the inhibition zones with polymyxin were never more than 4—6 mm wide, which would suggest that the diffusion technique employed is not recommendable with polymyxin.

TABLE 4

SENSITIVITY OF CONTROL STRAINS

Strain	New Antibiotics					Routine Antibiotics					
	Polymyxin B	Bacitracin	Neomycin	Terramycin	Gantricin	Sulfathiazole	Penicillin	Streptomycin	Aureomycin	Chloromycetin	Terramycin
E. coli 7337	+	+	++	+	±	±	—	—	+	—	+
E. coli 7395	+	+	++	++	±	±	—	—	+	+	+
E. coli 7326	+	+	++	++	±	±	—	±	+	±	+
Proteus 6630	—	+	—	±	±	—	—	—	—	—	—
Serratia	±	+	++	—?	±	—	—	—	—	+	—
Parat. B	+	+	++	+	±						
Microc. pyog. aur. 7401.....	+	++	++	±	—?	—	—	±	+	—	—
Microc. saprof. 7277	+	++	++	—	—	—	—	—	—	—	—
Microc. saprof. 7324	+	++	++	+	—	—	+	+	—	—	+
Enteroc. 7335.....	—	++	+	+	—	—	—	—	+	—	+

In the routine determinations: + sensitive
± less sensitive
— resistant

A number of the Pseudomonas strains therefore, may be, sensitive even though the inhibition zones do not reflect it.[1]

According to literature (8), with a dosage of 4—7 mg/kg/day the »polymyxin concentration» in the urine after 12 hours varied between 10 and 160 γ/ml. Albuminuria and casts seem to occur frequently although Brownlee et al did (2) not see histological changes or albuminuria in man; perhaps they used low concentrations. Polymyxin being bactericidal (5), this dosage, in the majority of cases, seems to produce a sufficient concentration in the urinary tract to cure Pseudomonas infection.

Bacitracin. — Bacitracin is generally not effective against gram-negative bacteria in the concentrations employed. According to literature (1) 50 units/ml did not inhibit Pseudomona growth. The Pseudomonas in fact required 187.5—375 units/ml in broth cultures to inhibit growth. In the dish experiments, surprisingly, two strains even showed great sensitivity to bacitracin. However, with these strains also, the inhibitory concentration is probably outside the limits of practical use.

Neomycin. — In the concentration employed neomycin proved to be distinctly the best of the antibiotics studied. According to literature (1) the growth of Pseudomonas is inhibited at the concentration 2.5 units/ml. In our investigations the inhibitory concentration in broth cultures proved to be 20.6 units/ml. (= 100 γ/ml. The manufacturers, Lääke Oy, give the effectiveness of the preparation at 206 units/mg). In the dish experiments all the strains were more or less sensitive to the corresponding concentration; 76 per cent were completely sensitive and another 13 per cent highly sensitive. Concentrations attained in practice (3) are 26—410 units/ml in the urine and 4—10 units/ml in the serum (in patients with kidney trouble even 16 units/ml). The effectiveness of purified neomycin B hydrochloride preparations is approx. 271 units/mg (3). This makes possible a concentration in the urine, and in many cases in the serum also, high enough to inhibit the growth of Pseudomonas. However, as neomycin has nephrotoxic properties, although to a milder degree than polymyxin (9), it is probably necessary to restrict its use to vital indications.

[1] According to Prissick (Am. J. Med. Sci. 1953:225:299) »The disc method indicates bacteriostatic, not bactericidal, concentrations». Thus, when polymyxin is bactericidal the disc method is not suitable for polymyxin sensitivity tests.

Terramycin. — The inhibitory terramycin concentration, according to literature, (1) is 100 γ/ml, which is confirmed by the present results. The corresponding concentration in dish experiments gave 56 per cent completely resistant, 23 per cent with slight growth-inhibiting effect, 18 per cent with partial inhibition of growth; the growth of a single strain was completely inhibited. With a daily dosage of 2 g the concentration in the urine varied between 100—300 γ/ml; (9) thus a sufficient concentration is sometimes achieved with a heavy dosage. However, Herrell et al. (4) state that the »terramycin content of the urine is not an accurate index as to its therapeutic effectiveness in infections of the urinary tract». It would seem, therefore that terramycin has no practical value against Pseudomonas infections.

Gantricin. — 80 γ/cc of gantricin gave complete inhibition of growth with a number of strains in the broth experiments. In the dish method, accordingly, 65 per cent of the strains were completely resistant, with 6 per cent a slight effect was achieved, and with 29 per cent a partial inhibition of growth. Concentrations of 60—100 μ/cc are reached, according to literature (7), depending on the quantity of urine. As complete inhibition is very rarely achieved with gram-negative rods with sulfonamides in sensitivity tests, although e.g. urinary tract infections in spite of this can be successfully treated with sulfonamide drugs, it would seem that nearly a

TABLE 5

STRAINS WHICH IN THE ROUTINE EXAMINATIONS DID NOT PROVE TO BE COMPLETELY RESISTANT, AND THEIR SENSITIVITY

Strain	New Antibiotics					Routine Antibiotics					
	Polymyxin B	Bacitracin	Neomycin	Terramycin	Gantricin	Sulfathiazole	Penicillin	Streptomycin	Aureomycin	Chloromycin	Terramycin
37	+	++	++	+	—	—	—	+	—	—	±
43	±	±	+	—	±	±	—	—	—	—	—
44	±	±	+	—?	±	±	—	—	—	—	—
46	—	+	++	—?	—?	—?	—	—	—	—	—?
51	—	±	+	—	—	—?	—	—	—?	—	—?
69	+	+	++	±	—	—	—	—	—?	—	—?

In the routine determination, symbol —? = retards growth.

third of the strains employed could perhaps have been controlled by an adequate gantricin dosage in the urinary tract.

As a rule, all the strains were completely resistant in routine determination. The six strains that were not completely resistant to the routine antibiotics are listed in Table 5. Strain 51, which routine determination found to be »retarded in growth» by sulfathiazole and terramycin, proved completely resistant to them in the test series. This is probably due e.g. to the dissimilarity of technique, in the first place to the smaller inoculation employed in the routine technique (6).

It was especially interesting to observe that the only strain (37) which proved sensitive to streptomycin in the routine determination was distinctly the most sensitive of all the strains to neomycin in the test series. The inhibition zone was some 20 mm.

CONCLUSIONS

In spite of the fact that gantricin did not prove to be completely growth-inhibiting with any single strain, and induced a partial inhibition of growth in a third of the strains only, it is probably recommendable for all Pseudomonas infections of the urinary tract, in view of the low toxicity of the drug. If it does not help large doses of terramycin can be tried; neomycin and polymyxin B must be confined to vital indications only. Considering the higher toxicity of polymyxin and the results achieved in the present investigation, well-purified neomycin preparations are probably to be preferred. In case of Pseudomonas sepsis neomycin is probably the best of the antibiotics available, perhaps combined with gantricin. Hence, wherever a local treatment of Pseudomonas infection is possible neomycin seems to be the antibiotic of choice.

SUMMARY

The material consisted of 60 Pseudomonas strains isolated from routine samples. Their sensitivity to polymyxin B, bacitracin, terramycin, neomycin and gantricin was investigated. Neomycin alone, in the concentrations employed, inhibited the growth of all the strains. 9 strains were completely resistant to polymyxin B, 6

strains to bacitracin, 34 strains to terramycin and 39 strains to gantricin.

The single Pseudomonas strain sensitive to streptomycin proved considerably more sensitive to neomycin than the other strains.

REFERENCES

1. Baron, A. L. Handbook of Antibiotics, Reinhold Publishing Corporation, New York 1950.
2. Brownlee, G., Buschby, S. R. M., and Short, E. I.: Ann. N. Y. Acad. Sci. 1949:*51:*952.
3. Duncan, G. G., Claney, C. F., Wolgamot, J. R., and Beidleman, B.: J.A.M.A. 1951:*145:*75.
4. Herrell, W. E., Heilman, F. R., and Wellman, W. E.: Ann. N. Y. Acad. Sci. 1950:*53:*448.
5. Kagan, B. M.: J. Lab. Clin. Med. 1951:*37:*402.
6. Kaipainen, W. J.: Ann. med. exper. et biol. Fenniae 1953 (in press).
7. Langeman, H.: Schweiz. Med. Wschr. 1950:Nr. *4:*78.
8. Schoenbach, E. B., Bryer, M. S., and Long, P. H.: Ann. N. Y. Acad. Sci. 1949:*51:*987.
9. Schoenbach, E. B., Bryer, M. S., and Long, P. H.: Ann. N. Y. Acad. Sci. 1950:*53:*245.

FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

RESISTANCE TO ADRENALINE DURING PREGNANCY

AN ELECTROCARDIOGRAPHIC STUDY OF THE TOXICITY OF ADRENALINE IN GUINEA-PIGS

by

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The thyroid gland has since the earliest times been known to undergo a typical increase in size during pregnancy. Serum precipitable or protein-bound iodine is known to increase during pregnancy (14). This is usually accompanied by an increase in the serum thyroxine (7). It is furthermore known that in both therapeutic and toxic doses thyroxine greatly enhances the activity of adrenaline (3, 17). Unexpected sudden deaths have occurred during operations on toxic goitre when anaestetics containing adrenaline have been used (24).

On the other hand, it is interesting to note that the ability of blood to decompose histamine is increased many thousandfold during pregnancy (1, 32). In many of their pharmacological properties histamine and adrenaline are antagonistic. Eichler and Barfuss (10) and Staub (28), have established that histamine is liberated by adrenaline in physiological conditions in both man and rabbit. Dale (6), Burn and Dale (4) and Mackay (18) found that injection of histamine effects increased suprarenal secretion of adrenaline. If the increased histaminolytic ability observed during pregnancy leads to an increase in the ability of the organism to utilize larger amounts of histamine to counteract the effects of adrenaline a decreased adrenaline toxicity should result. Eichler and

Barfuss (10) have, however, expressed the opinion that histamine increases sensitivity to adrenaline in cats, but on the other hand, in pregnant guinea-pigs the toxicity of histamine is only one-fourth of the normal (25).

Owing to the placental circulation, the blood circulation of pregnant individuals differs from the normal. The placenta is a nerve free organ (26). Furthermore, the increased production of folliculine hormone during pregnancy has been found to increase the peripheral blood circulation (22). On the other hand, it is well established that tone of the smooth muscles is depressed by progesterone.

On the basis of the above, we were led to the assumption that the toxicity of adrenaline differs in pregnant and non-pregnant individuals. This has been investigated with guinea-pigs by determining the lethal dose of adrenaline on continuous infusion and recording the changes in heart function electrocardiographically during the infusion. It has previously been established that even small doses of adrenaline effect typical changes in cardiac function that can be recognized in the electrocardiograms (9, 21).

The fact that the increase in blood pressure induced by injection of adrenaline is smaller before than after delivery in women is according to Barthel (2) due to the general instability of the autonomic nerve system. Ovariectomy did not increase the adrenaline response; on the contrary, it led to a weaker response.

MATERIAL AND METHODS

The animals were female guinea-pigs that belonged to the same strain and had grown under identical conditions. Control animals were virgins. The pregnant animals were considered to be within two weeks of labour. The animals were freely given hay, swedes, oats and water before the experiment.

The animals were anesthetized with 25% urethane solution (2 g of urethane per kg subcutaneously). Ether was not used at any stage during the experiment. The top of the operating table was of insulating material. About one hour after the administration of urethane, the jugular vein was prepared and furnished with cannula for the infusion of adrenaline solution. Adrenaline base was dissolved in physiological salt solution using the requisite amount of hydro-

chloric acid to give a concentration of 20 mg per cent. In this work, the doses of adrenaline refer to the base. The infusion was conducted dropwise and as accurately as possible to give 0.9 ml of the above solution (0.18 mg of adrenaline base) per kg body weight per minute until breathing ceased. The electrocardiograms were recorded with a Triplex (Elmquist system) electrocardiograph. Standard leads I, II and III were used throuhout the experiments. Recordings were made after 10, 20, 40, 60, 90 and 120 seconds from the beginning of the infusion and thereafter at minute intervals.

TABLE 1

TOXICITY OF INFUSED ADRENALINE (0.18 MG/KG/MIN.) IN PREGNANT GUINEA-PIGS. URETHANE (2 G/KG WAS INJECTED SUBCUTANEOUSLY ONE HOUR BEFORE THE INFUSION OF ADRENALINE. THE UPPER NUMBERS ARE LETHAL DOSE VALUES, THOSE IN BRACKETS GIVE THE RANGE

	No. of Animals	Adrenaline		Body Weight g	Lung Weight	
		mg/kg	Infusion Time in min.		g	% of Body Weight
Non-Pregnant	13	1.9 (0.8—2.3)	8.3 (4—11)	531 (400—550)	7.9 (5.0—10.5)	1.49
Pregnant [1] I	10	3.7 (2.2—4.6)	20.9 (11—24)	944 (800—1000)	8.0 (5.5—11.0)	0.85
Pregnant [1] II	10	4.6 (3.4—5.9)	20.9 (11—24)	768 (550—935)	8.0 (5.5—11.0)	1.04 [2]

[1] I and II refer to the same group of pregnant animals, but the results II have been calculated by using as body weights the weights obtained by subtracting the weights of the foetus and placenta from the actual weight.

[2] The standard deviation of the mean of the relative weights of the lungs (in %) is 0.14 for the non-pregnant animals and 0.09 for pregnant animals (II). The deviation of the difference between the average values for these two groups is 0.16, and hence the difference is significant.

RESULTS

It is evident from Table 1 that the lethal dose of adrenaline for the non-pregnant animals is 1.9 mg/kg, whereas the corresponding figure for the pregnant animals is 3.7 mg/kg, or twice as large. If the lethal dose is calculated with respect to the weight after subtracting the weight of the foetus and placenta, the difference is even larger. The relative weight of the lungs as a percentage of the body weight is largest for the non-pregnant animals, 1.49%, and smallest for

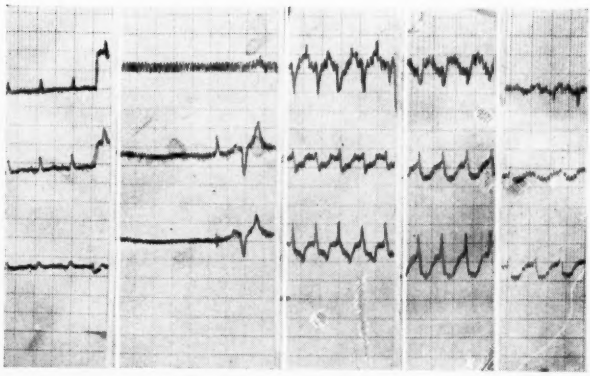
the pregnant animals, 0.85%. The relative weight of the lungs of the pregnant animals is 1.04% of the reduced weight.

General tonic and partly clonic convulsions were observed during the infusion of adrenaline in both animal groups. Convulsions were more frequent among the non-pregnant animals and after respiratory embarrassement these were particularly strong. No convulsions or only weak ones were observed in the pregnant group. A similar deep breathing was not noted in the latter group as in the non-pregnant group. After the respiration was ceased blood-containing froth issued from the nostrils of most of the non-pregnant animals, but this occurred in only a few cases in the pregnant group.

In post-mortem examination slight edema and hemorrhage were observed in the lungs of both groups, but these changes seemed to be less marked in the pregnant groups. The right half of the heart contained much blood. When the chest was opened during the first few minutes after respiration ceased, the heart was still observed to beat; also the hearts of the foetus continued to beat at this stage. Five to ten minutes after then infusion was begun, strong movements of the foetus were noted which continued one to several minutes, but these had in all cases ceased before respiration came to an end.

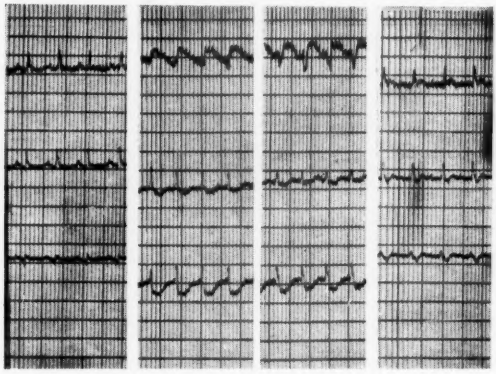
Electrocardiographic Changes.[1] — No differences in cardiac frequency were observed between the two animal groups. Under urethane anesthesia, the heart rate was generally 300 per minute. Immediately after the adrenalin infusion was begun, a marked bradycardia was noted which was in most cases followed by extrasystolic heart contraction. In some animals the latter were of bigeminal form, but in most cases they occurred after irregular intervals and the time at which they began varied. The pulse had become even with a frequency of approximately 480 beats per minute within one minute after the beginning of the infusion. During the next few minutes the pulse slowed down gradually and was about 390 beats per minute when respiration ceased. The electrocardiograms of the groups differed from each other primarily in the form of the ST interval and in the direction of the electrical axis. The initially normal or in a few cases levo-inclined electrocardiogram

[1] We are indebted to Dr. A. Telkkä for valuable criticism in the interpreting of the electrocardiographic changes.



Before 20 sec. 40 sec. 90 sec. 4 min.

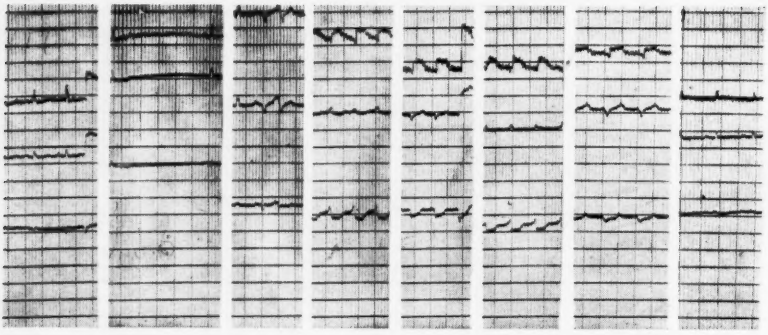
Fig. 1. — Non-pregnant. Respiration ceased 8 minutes after infusion was begun. Time from the beginning of the adrenaline fusion has been noted accordingly.



Before 1 min. 2 min. 6 min.

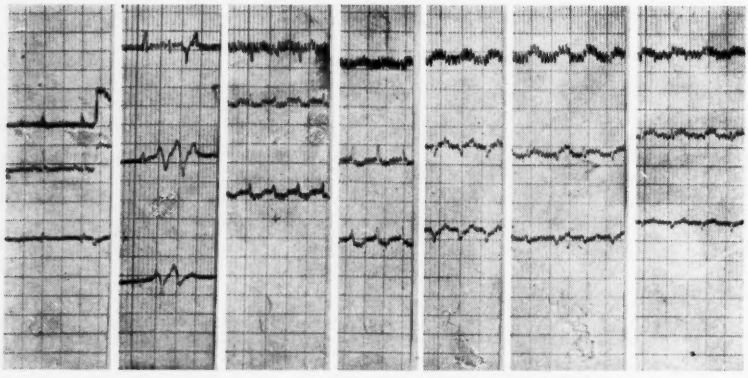
Fig. 2. — Non-pregnant. The last electrocardiogram recorded after respiration ceased.

had in the non-pregnant animals after 40—60 seconds transformed into the dextro-form and this form prevailed as long as infusion was continued (Figs. 1 and 2). Deviation of the electrical axis to the right was observed in all animals for at least some time. Dextrograms were found in only one-half of the pregnant animals and even then during only a short period. (Figs. 3 and 4). With some of the animals the levogram or normogram alternated with the dextrogram. This was not noted in the non-pregnant group. Along with the deviation of the electric axis to the right, a strong elevation of



Before 10 sec. 20 sec. 2 min. 3 min. 4 min. 11 min. 19 min.

Fig. 3. — Pregnant. Respiration ceased after 21 minutes.



Before 20 sec. 1 min. 1⅓ min. 2 min. 4 min. 6 min.

Fig. 4. — Pregnant. Respiration ceased after 15 minutes.

the ST interval was noted in tracing I and a decrease in tracing III; ST II underwent a slight depression. These changes were much more pronounced in the non-pregnant group, and there was hence no difficulty in deciding to which group the animal belonged. The electrocardiograms recorded after respiration ceased were in both groups very similar to those taken before the infusion.

It was difficult to evaluate the P waves in the recorded electrocardiograms, but a sinus rhythm was, however, prevalent except at the beginning when the occurrence of extra systoles obscured the waves. Very little can with certainty be said about the QRS complex.

DISCUSSION

Dornhorst and Young (8) were not able to establish that transfer of adrenaline occurs from mother to foetus or *vice versa*; nor that the changes effected in the maternal circulation following injection of adrenaline or noradrenaline led to a reduction in the blood supply to the maternal side of the placenta and a consequent asphyxia of the foetal heart. The dose of adrenaline was actually much greater in the present work, but if it is assumed that adrenaline does not enter the foetal circulation, the difference between the total weight and the weight of the foetus and placenta should be taken as the body weight of the pregnant animals. In both groups respiration ceased before the heart stopped, which is typical of adrenaline toxicity in guinea-pigs (11). Hemorrhagic lung edema was of low degree when the relative weights of the lungs are compared with, for example, the data of Schmiterlöw and Wessman (27). The latter authors found that the weights of the lungs of animals that died after rapid intravenous injection of adrenaline (adrenaline hydrochloride, 0.125—0.5 g/kg) was on the average over 2 per cent of the body weight (in the controls 0.76%). The weight of the lungs of animals that had withstood the injection of adrenaline was less than 1.57 per cent of the total body weight. In the present series the figures corresponding to the preceding percentage were 1.49 per cent for the non-pregnant group and 0.85 (1.04) for the pregnant group. The lung edema was relatively weak and death probably followed of central respiratory or cardiac failure. Although the heart still functioned weakly after respiration ceased, it is not certain that the contractions were adequate since there was much blood in the right heart. Narcosis is believed to prevent the development of pulmonary edema by adrenaline, but barbiturates cause no inhibition in near anesthetic doses (30). Likewise urethane does not prevent acute pulmonary edema due to adrenaline (23). The marked bradycardia initially observed in the electrocardiograms is considered to result from reflexively increased blood pressure (16). It is known that even small doses of adrenaline effect ventricular rhythms following sensibilization by, for example, chloroform and cyclopropane and may often lead to death as a result of ventricular fibrillation. In our experiments the premature ventricular contractions following bradycardia completely dis-

appeared within a short time and no ventricular fibrillation was observed. The more marked changes in the electrical axis and the ST interval in the non-pregnant group suggest a relatively more pronounced exertion of the right ventricle. The most probable cause of this may be the relatively limited blood circulation in the coronary arteries. The studies of Melville and Lu (20) have shown that adrenaline effects an increased coronary blood circulation but this is not adequate enough to take care of the increased work of the heart. If the increase in blood pressure were not as large in the pregnant as in the non-pregnant animals, the probability of development of acute cardiac insufficiency in the right heart would be smaller in the pregnant group. (Evidently the relaxating of particularly the smooth muscles during pregnancy alters in part the blood circulation conditions). The placenta possesses no nerve system and although many investigators have shown that adrenaline effects a contraction of the vessels of the placenta, it is probable that as the peripheral resistance increases following the administration of relatively large doses of adrenaline the same degree of vasoconstriction does not occur in this organ as in other organs. The work of the heart does not hence increase as much as in non-pregnant individuals. It may be considered that there is in the systemic circulation an organ which decreases the pressure and resistance and which in many blood circulatory properties resembles the lungs (12). Burwell (5) has also explained the temporary reduction in pressure in artificially induced hypertension during late pregnancy to be due to the change in haemodynamic condition resulting from the presence of the placenta.

In the introduction reference was made to the possible influence of histamine. Oestradiol augments the histamine content of the blood, liver and lungs in the guinea-pig (15). It is possible that at the time of death the tissues of the animals have a high adrenaline content. The placentas of guinea-pigs and rabbits have a high contant of mono-oxidase (31) and defibrinated blood of pregnant women contains variable amounts of certain mono- and diamino-oxidases (29).

The amount of adrenaline infused during one minute corresponds to the average lethal dose in intravenous administration (27, 33) and hence the elimination of such a dose does not seem possible during this short period of time. According to Massons and Valde-

casas (19), collapse from adrenaline is determined more by the duration of hypertension and ischemia than by the size of the dose. Consequently the most significant factor is the duration of the infusion. The present data show that the adrenalin tolerance of pregnant guinea-pigs is approximately twice that of non-pregnant guinea-pigs whether this is judged on the basis of infusion time or dose size.

SUMMARY

The toxicity of adrenaline administred by continuous intravenous infusion has been studied in pregnant and non-pregnant female guinea-pigs. The lethal dose for non-pregnant animals was found to be 1.9 mg/kg on the average and the time of infusion 8.3 minutes. The corresponding figures for the pregnant animals were 3.7 mg/kg and 20.9 min. Strong convulsions were observed in the non-pregnant animals, but in the pregnant animals the convulsions were either not observed or were very weak.

The electrocardiograms recorded during the infusion initially revealed bradycardia and extrasystoles. This was followed in the non-pregnant animals by a deviation of the electric axis to the right, a marked elevation of the ST segment in lead I and depression in leads II and III, especially in lead II. In the pregnant guinea-pigs, these changes were very slight or were not observed at all.

The possible causes of this difference in behaviour have been discussed.

REFERENCES

1. Ahlmark, A.: Acta Physiol. Scandinav. 1945:*9:*suppl. 28.
2. Barthel, E.: Diss. Offenbach on the Main 1926.
3. Bergwall, Å., and Kuschinsky, G.: Arch. f. exper. Path. u. Pharmakol. 1931:*162:*169.
4. Burn, J. H., and Dale, H. H.: J. Physiol. 1926:*61:*185.
5. Burwell, C. S.: Am. J. M. Sc. 1938:*195:*1.
6. Dale, H. H.: Brit. J. Exp. M. 1920:*1:*103.
7. Danowski, T. S., Gow, R. C., Mateer, F. M., Everhart, W. C., Johnston, S. Y., and Greenman, J. H.: Proc. Soc. Exper. Biol. & Med. 1950:*74:*323.
8. Dornhorst, A. C., and Young, I. M.: J. Physiol. 1952:*118:*282.
9. Douglas, A., Gelfand, B., and Shookhoff, Ch.: Am. Heart J. 1937: *14:*211.

10. Eichler, O., and Barfuss, F.: Arch. f. exper. Path. u. Pharmakol. 1940:*195:*245.
11. Elliot, T. R.: J. Physiol. 1905:*32:*401.
12. von Euler, U. S.: J. Physiol. 1932:*74:*271.
13. Goetsch, E.: Long. Island Med. J. 1922:*16:*155.
14. Heinemann, M., Jonson, C. E., and Man, E. B.: J. Clin. Investigation. 1948:*27:*91.
15. Herschberg, A. D.: Compt. rend. Soc. biol. 1951:*145:*512.
16. Heymans, C.: Arch. internat. de Pharmacodyn. et de thérap. 1929:*35:* 307.
17. Krayer, O., and Sato, G.: Arch. f. exper. Path. u. Pharmakol. 1928: *128:*67.
18. Mackay, M. E.: J. Pharmacol. 1929:*37:*349.
19. Massons, J., Valdecasas, M. A., and Valdecasas, F. G.: Rev. españ. fisiol. 1945:*1:*107. Cit. Hartman, F. A., and Brownell, K. A.: The Adrenal Gland, Lea & Febiger, Philadelphia 1949.
20. Melville, K. J., and Lu, F. C.: J. Pharmacol. 1950:*99:*286.
21. Milles, G., and Smith, P.: Am. Heart J. 1937:*14:*198.
22. Mühlbock, O., Knaus, H., and Tscherne, E.: Die Weiblichen Sexualhormone in der Pharmakotherapie, Hans Huber, Bern 1948.
23. Paasonen, M. K.: Ann. med. exper. et biol. Fenniae, 1953:*31:*Suppl. 7.
24. Paus, B.: Nord. Med. 1947:*34:*867.
25. Pylkkö, O. O., and Vartiainen, A.: Acta Pharmacol. et Toxicol. 1947: *3:*97.
26. Schmitt, W.: Ztschr. f. Biol. 1922:*75:*19.
27. Schmiterlöw, C. G., and Wessman, C. G.: Acta Physiol. Scandinav. 1951:*23:*31.
28. Staub, H.: Schweiz.med.Wschr. 1946:*76:*818; Experientia 1946:*2:*29.
29. Stefano, V.: Boll. Soc. Ital. Biol. Sper. 1947:*23:*95.
30. Stone, C. A., and Loew, E. R.: Proc. Soc. Exper. Biol. & Med. 1949: *71:*122.
31. Thompson, R. H. S., and Tickner, A.: Biochem. J. 1949:*45:*125.
32. Verle, E., and Effkemann, G.: Arch. Gynäkol. 1940:*170:*82.
33. Vigran, J. M., and Essex, H. E.: Am. J. Physiol. 1950:*162:*230.

FROM THE STATE SERUM INSTITUTE, HELSINKI

OCCURRENCE OF BLOOD GROUP P IN FINLAND

by

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(Received for publication May 6, 1953)

The blood group P was discovered by Landsteiner and Levine (7) in 1927. The agglutinin corresponding to this method they found in the immune serum of rabbit. The so-called extra-agglutinin I found previously in human serum, gave reactions similar to those of the above-mentioned immune serum, and agglutinins analogous in function were later established in the sera of horse, cow, pig and sheep (8). Landsteiner and Levine (9) found P antigen to vary considerably in intensity in different persons. Through their investigations they also became positive about the heredity nature of the P property.

Dahr (2) proved that the P property is inherited according to the mendelian law, bound to a simple pair of allelic genes; he used the symbols P and p to denote this pair of genes. Several other investigators have confirmed the heredity theory (6, 12, 5).

Henningsen (3) has published a sensitive technique, largely founded on Jonsson's method of determination, by means of which even weak P properties can be reliably demonstrated. He did not consider it possible to make a sharp distinction between P properties of different strengths (4). To judge from his investigations of families he concludes that the changes in the intensity of the P property can be accounted for in no other way than by the existence of a minimum of three positive genes, all dominant vis-à-vis the negative gene. P+ × P— parents cannot beget a child in whom the P property is more strongly developed than with a positive parent (4). The antigen is not fully developed in children at the

moment of birth. If the technique employed is sufficiently sensitive anti-P agglutinins (3) can be demonstrated in the sera of most P-negative persons.

The frequencies of P-positive and -negative individuals vary in different races. The following table gives the results of investigations conducted in this respect:

TABLE 1[1]

THE DISTRIBUTION OF THE P GROUPS

	Total	P+	P—	Gene P	Gene p
Cologne	6478	4769	1709		
Dahr, 1942.....		73.62 %	26.38 %	0.4864	0.5136
Jungmichel, 1942	1586	1198	388		
		75.54 %	24.46 %	0.5054	0.4946
Copenhagen	506	413	93		
Andresen, 1941		81.62 %	18.38 %	0.5713	0.4287
Copenhagen	2345	1849	496		
Henningsen, 1949		78.85 %	21.15 %	0.5401	0.4599
Stockholm	1089	831	258		
Jonsson, 1947		76.31 %	23.69 %	0.5133	0.4867
Cairo (Mixed inhabitants)	1000	775	225		
Moharram, 1942		77.50 %	22.50 %	0.5257	0.4743
New York (Whites)....	237	176	61		
Wiener & Unger, 1944..		74.26 %	25.74 %	0.4927	0.5073
New York (Negroes) ..	73	71	2		
Wiener & Unger, 1944..		97.26 %	2.74 %	0.8345	0.1655
London	500	370	130		
Sanger et al., 1949		74.00 %	26.00 %	0.4901	0.5099
Prague	181	138	43		
Sanger et al., 1949		76.24 %	23.76 %	0.5126	0.4874
Firenze	250	205	45		
Chiodi, 1948		82.00 %	18.00 %	0.5757	0.4243

The writer aims at throwing light upon the distribution of blood group P in Finland. Two series of 150 and 550 Finnish Red Cross blood donors have been investigated for this purpose.

[1] From R. R. Race and Ruth Sanger, *Blood Groups in Man*, Blackwell, Oxford, 1950, p. 66, Table 15.

MATERIAL AND METHODS

A difficulty facing P blood group determination is to distinguish between slightly P-positive individuals and the negative. Strong human sera containing anti-P agglutinins are very rare. It is easier to find serviceable animal sera.

Animal sera have usually been prepared by the slide method. Dahr and Jungmichel used 15' incubation time at room temperature, reading the reactions with the naked eye. Andresen (1) used a similar technique for human sera but with incubation at + 6°C.

Jonsson's technique, on which Henningsen's method of determination largely depends, is as follows: both horse and human sera are used. When horse serum is used the investigation is made on slides at + 18°C and the reactions are read with the naked eye and microscopically at 5' intervals up to 35'. This enables observation of the agglutination rate and intensity. When human serum is used the investigation is carried out in tubes which are left in a refrigerator over night and the reactions are read with the naked eye. In addition an absorption test is made on each sample, using human serum. Henningsen employed various human sera in agglutination and absorption tests.

The writer had at his disposal two horse sera, R/559 and R/569 and one human serum, LN. The titres of these sera with the strongest P-positive red blood cells were:

R/559	+ 18°C slide method	1 : 12
	+ 6°C in test tubes	1 : 16
R/569	+ 18°C slide method	1 : 8
	+ 6°C in test tubes	1 : 16
LN	+ 18°C in test tubes	1 : 8
	+ 6°C in test tubes	1 : 16

The horse sera were absorbed with P-negative A_1, B_1 and O red blood cells both at room temperature and in a refrigerator. The human serum belonged to group A and was absorbed with P-negative B cells at room temperature and in a refrigerator.

The tests were performed in two parts.

Part I. — 150 samples. Agglutination tests were made with all the above-mentioned sera. Horse sera were prepared by Jonsson's technique. The dilution of the R/559 serum used was 1: 2 and that

of the R/569 serum 1: 1 ½. One drop of the 1% red cell suspension to be tested was added to two drops of the serum. Human LN serum was used undiluted and the tests were performed in 5 × 50 mm tubes in the following manner: 1 drop of red cell suspension was added to 1 drop of serum and the tubes were allowed to stand for 2 hours at + 6°C. The reactions were read by smearing the sedimented red cells carefully on the slide from a Pasteur pipette, followed by miscroscopic examination. The absorption test was performed with the R/559 horse serum as follows: 1 drop of packed red cells was added to 3 drops of 1:2 diluted serum, the tubes were allowed to stand for 3 hours at + 6°C during which period they were agitated three times. The serum on top of the red cell sediment was tested with medium-strong P-positive red cells in 1% suspension, in exactly the same way as for the agglutination test, using the slide technique according to Jonsson. Reaction rates and intensities were used to interpret the results. Control tests with known medium-strong P-positive and P-negative red blood cells were appended to all determinations.

Part II. — 550 samples. The agglutination tests were made in tubes with both horse and human sera in exactly the same way as in Part I with human serum. The only difference was that incubation time was changed to 4 hours. Additional absorption of horse sera was necessary for this technique. The serum dilutions used were the same as in Part I. In addition to human LN serum the absorption tests were made with R/559 horse serum as follows: 1 drop of packed red cells was added to 2 drops of serum, the absorption solution was kept in a refrigerator over night and on the following day the serum on top of the sediment was tested with medium-strong P-positive red cells as in the above-mentioned agglutination test. Control tests were added to all determinations. All questionable results were controlled again by means of the same agglutination and absorption technique to eliminate reading mistakes.

RESULTS

The absorption tests were given a decisive role in the assessment of the results. In the agglutination tests all very weak or completely negative samples were entered as P-positive in so far as it was possible to ascertain the loss of the anti-P antibody in absorbed serum.

Part I of the study established 42 samples out of 150, or 28.0%, as P-negative. In absorption tests 4 samples proved positive (2.7%); they gave a negative result in agglutination tests with both human and horse sera. One sample (0.7%) which agglutinated with horse serum only likewise turned out to be positive. Six samples (4.0%) which gave a weakly positive result with horse serum and a negative result with human serum proved P-negative in the absorption tests.

In Part II, covering 550 samples, 140 or 25.5% were ascertained to be P-negative. In absorption tests 8 samples (1.5%) proved positive; they had given a negative agglutination reaction with all sera. Two samples (0.4%), which agglutinated distinctly with human serum but always gave a negative result with horse sera, were likewise entered as positive. Horse sera gave 1.5% more positive agglutination results than human serum.

The ABO-groups of samples tested in Part I were selected since human LN serum had not yet been absorbed in that phase. The samples of Part II were unselected. This is why the following table gives the distribution of ABO- and P-blood groups only of the 550 blood donors.

TABLE 2

ABO-groups	Number	%	P+	P—	% of P—
A	240	43.6 ± 3.2	175	65	27.1 ± 2.9
O	179	32.6 ± 3.5	135	44	24.6 ± 3.2
B	86	15.6 ± 3.9	67	19	22.1 ± 4.5
AB	45	8.2 ± 3.6	33	12	26.7 ± 6.7
Total	550	100.0	410	140	25.5 ± 1.9

DISCUSSION

According to Streng (11) the ABO-groups are distributed in the Finnish people as follows: A 41.9%, O 32.5%, B 18.2% and AB 7.4%. An examination of the breakdown of the ABO-group in the table shows the blood donors examined to be fairly representative of an unselected Finnish material. The frequency fluctuation of P-negative individuals appearing in the different ABO-groups lies within the limits of the mean error in such a small material as this.

Comparison of the results arrived at in the present study with

those presented in Table 1 shows the number (25.5%) of P-negative individuals to be greater than in Italy and Denmark but of approximately the same size class as in Sweden, England and Germany.

The reason why the slide method used for horse sera in Part I was changed in Part II for the test tube method is first and foremost the very generous presence of rouleaux formations. Agglutinins obtained by means of the tube technique were, in addition, more solid, the reactions more parallel to the reactions given by human sera and the method was more time-saving. The lengthening of the incubation period in the agglutination test to 4 hours and in the absorption tes to 18 hours made the results more reliable. Absorption tests on both human and horse sera were considered as absolute necessary.

SUMMARY

(1) The writer gives a short general survey of earlier P blood group studies.

(2) The determination techniques used in the work are introduced and the test tube method and incubation at + 6° C are considered better and also more time-saving for the sera employed.

(3) The results of the work — the material studied contained 25.5.% P-negative individuals — are presented.

REFERENCES

1. ANDRESEN, P.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 100:*429:* 1941.
2. DAHR, P.: Technik der Blutgruppen und Blutfaktorenbestimmung. 5 Auflage. Georg Thieme, Leipzig. 1950.
3. HENNINGSEN, K.: Acta Path. et Microbiol. Scandinav. 1949:*26:*639.
4. HENNINGSEN, K.: ibid. 1949:*26:*769.
5. JONSSON, B.: Social-Medicinsk Tidskrift 1947.
6. JUNGMICHEL, G.: Ztschr. f. d. ges. gerichtl. Med. 1942:*36:*259.
7. LANDSTEINER, K., and LEVINE, P.: J. Exper. Med. 1928:*47:*757. Cited by WIENER, A. S.: Blood Groups and Transfusions, Charles C. Thomas, Springfield Ill. 1946.
8. LANDSTEINER, K., and LEVINE, P.: J. Immunol. 1931:*20:*179.
9. LANDSTEINER, K., and LEVINE, P.: ibid. 1930:*18:*87.
10. RACE, R. R., and SANGER, R.: Blood Groups in Man. Blackwell. Oxford. 1950.
11. STRENG, O.: Acta Soc. Med. Fenn. Duodecim. A, T. 1935:*17:* F3.
12. WOLFF, E.: Statens offentliga Utredningar. 1946:49.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE ANTIBIOTIC SENSITIVITY OF MICROCOCCI ISOLATED FROM PYODERMA

by

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(Received for publication May 12, 1953)

The use of antibiotics is constantly increasing in the treatment of pyoderma. The most frequent cause of pyoderma known is *Micrococcus pyogenes v. aureus*. Its antibiotic sensitivity is apparently (2, 3, 7) decreasing, particularly to penicillin but also to other antibiotics. In order to verify the development in our country I have collected the results of sensitivity tests performed during 1951 and 1952—53. A disc technic was used (5).

Material and Results. — The sensitivity tests of micrococci were made from samples sent to us from the University Dermatological Clinic during 1951 and 1. 9. -52 — 30. 4. -53. (Table 1)

TABLE 1

THE NUMBER OF RESISTANT MICROCOCCUS STRAINS IN SAMPLES FROM THE UNIVERSITY DERMATOLOGICAL CLINIC DURING 1951 AND 1952—53 (8 MONTHS)

	Sulfathiazole	Penicillin	Streptomycin	Aureomycin	Chloromycetin	Terramycin
1951 111 strains, resistant per cent	50	36	21	9	3	3
1952—53 166 strains, resistant per cent	80	57	36.5	23	42	20.5

In addition, there were samples from the University Dermatological Clinic (D.C.) and the Dermatological Department of the Central Military Hospital (C.M.H.) sent to us during 1. 9. -52 — 30. 4. -53 in which group the coagulase-positive *Micrococcus pyogenes v. aureus* were analysed separately. (Table 2)

TABLE 2

THE NUMBER OF RESISTANT STRAINS IN SAMPLES FROM THE UNIVERSITY DERMATOLOGICAL CLINIC AND THE DERMATOLOGICAL DEPARTMENT OF THE CENTRAL MILITARY HOSPITAL DURING 1952—53 (8 MONTHS)

	Sulfathiazole	Penicillin	Streptomycin	Aureomycin	Chloromycetin	Terramycin
University Clinic						
All strains (166) resistant per cent	80	57	36.5	23	42	20.5
Coagulase positive (147) resistant per cent	80	59	38	23	40	21
Military Hospital						
All strains (91) resistant per cent	87	58	31	13	30	10
Coagulase positive (76) resistant per cent	89.5	60.5	33	14.5	34	12

The D.C. material consisted of samples from both in-patients and out-patients. The patients were of various ages. Some, perhaps most of them, lived in Helsinki, but many came from rural communities. Many of the patients had received antibiotic treatment before. The C.M.H. material consisted principally of samples from young adults (19—21 years). A definitely greater proportion of them were from rural communities than in the University material. They had, in general, not received antibiotics before, except perhaps penicillin and sulfonamides.

Table 3 gives the percentage of resistant strain of micrococci in the United States (1.), Denmark (6.) and in our material (coagulase-positive).

It is interesting that the percentage of penicillin-resistant strains seems to be the same in Finland as in other countries. The percentage of sulfonamide-resistant strains in the D.C. material was about the same as in Denmark, but the clearly greater percentage of Finnish strains resistant to the so-called broad-spectrum anti-

TABLE 3

THE NUMBER OF RESISTANT MICROCOCCUS STRAINS IN THE UNITED STATES, DENMARK AND FINLAND (COAGULASE POSITIVE)

Resistant strains per cent		Sulfathiazole	Penicillin	Streptomycin	Aureomycin	Chloromycetin	Terramycin
United States	1951		59		33	15	
Denmark	1952	78	59	17	1	4	2
Finland							
D.C.	1952—53	80	59	38	23	40	21
C.M.H.	1952—53	89.5	60.5	33	14.5	34	12

biotics is surprising. Naturally there are dissimilarities in technics; in Denmark, for example, the sensitivity to streptomycin has been tested with a greater streptomycin concentration than we use, but technical dissimilarities alone hardly explain such differences in sensitivity.

Most conspicuous was the rise of resistance to chloromycetin, since it has been used only in exceptional cases in dermatological clinics. Perhaps it was due to a cross-resistance, and caused primarily by an increased resistance to the broad-spectrum antibiotics in general. (4)

There were differences in the sensitivity of the micrococci originating from our two hospitals (Table 2), partly due to the dissimilarity of patients. But the frequent occurrence of sulfonamide-resistant strains in the C.M.H. may be attributed to the greater use of sulfonamides there. In the C.M.H. there was no restraint in the use of the sulfonamides and penicillin, but the broad-spectrum antibiotics could be used only in exceptional cases with deliberation »in casu» on the basis of sensitivity tests, and on each occasion the antibiotic had to be ordered specially from the Army Medical Board. Streptomycin and the broad-spectrum antibiotics were freely used in the D.C. In these circumstances it is no wonder that the micrococci from the D.C. were more resistant to broad-spectrum antibiotics.

SUMMARY

The number of resistant strains of *micrococcus pyogenes v. aureus* seems to be increasing also in Finland. There are about 60 per cent of penicillin-resistant strains, as in other countries. But there are also many aureomycin-, chloromycetin- and terramycin-resistant strains.

REFERENCES

1. Altmeier, W. A., and Culbertson, W. R.: J.A.M.A. 1951:*145:*449.
2. Banks, H. S.: Lancet 1952:*I:*560.
3. Clarce, S. K. R., Dalgleish, P. G., and Gillespie, W. A.: Lancet 1952:*I:*1132.
4. Kaipainen, W. J.: Ann. Med. Exp. Biol. Fenn. 1951:*29:*suppl.1.
5. Kaipainen, W. J.: Ibid 1953 (in press).
6. Lund, E.: Acta Path. Microbiol. Scand. 1952:*31:*281.
7. Rountree, P. M., and Thomson, E. F.: Lancet 1952:*II:*262.

FROM THE STATE SERUM INSTITUTE, HELSINKI, AND HELSINKI CITY HEALTH DEPARTMENT

INFLUENZA EPIDEMIC IN HELSINKI IN 1953

by

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(Received for publication June 6, 1953)

The following is a report on the influenza epidemic in Helsinki in 1953. A comparison is made between the incidence of influenza in children attending kindergartens, children at primary schools, and adults. The reliability is assessed of the data on persons of different ages as illustrative of the onset and progress of the influenza epidemic. Some information is also introduced on deaths attributed to influenza. Finally, some results are given of the laboratory investigations made of the epidemic.

Attack Rate of Influenza in Kindergarten and Primary School Children, and Adults. — The collection of data on cases of illness resembling influenza in certain population groups was commenced in the autumn of 1952. The data are based on the illness reported as the cause of absence. Included in this connection are firstly, sudden influenza-like illnesses accompanied by fever either with or without respiratory symptoms. Another group reported is the pneumonias. The subject observed have been adults, varying in numbers from week to week between 17,412 and 21,904. The adults under observation have mainly been employed in public transport, the metal industry, business firms and the postal service.[2] Schoolchildren observed have been pupils of primary schools of the City

[1] Aided by a grand from the Sigrid Juselius Foundation.

[2] The writer is indebted for information received to Drs. M. Rouhunkoski, L. Ekman, U. Rinne, C. O. Biström, P. Sumari, L. E. Tötterman, L. Noro, J. Hakkila, Miss Hankama, public health nurse, and Mr. W. Stepanof, welfare officer.

of Helsinki.[1] The number of pupils in the schools under observation totalled approximately 17,500 with small variations. The weekly number of kindergarten pupils under observation was in the region of 538—2,339. The weekly attack rate has been calculated with reference to the variation in the number of subjects under observation. However, the figures obtained from different groups probably do not give the actual situation prevailing at a given moment as there has been some discrepancy in the principles of assessment owing to the large number of persons participating in the work. On the other hand the successive figures of the same groups probably compare fairly well with one another. The breaks in the curves for school children and kindergarten pupils are caused by holidays.

All told it can be considered that the population groups under observation accounted for nearly 10% of the inhabitants of the city (384,521 persons in 1952).

The curves illustrating the incidence of the disease, obtained in the way mentioned above, are given in Graph No. 1. The time on each side of the epidemic proper has been described at some length in order to make clear the general nature and reliability of the curves. Vertical lines have been drawn at the dates of the first and last positive garglings taken in the course of the spring. The area between these vertical lines may be considered the period of the influenza epidemic.

The curve for adults is very even both before and after the epidemic period. The kind of »illness» or »absence for other reasons» illustrated by the basic level of the curve can only be guessed at. Values showing weekly incidence of over 1 per cent are encountered only during the epidemic. The curve for school children on the other hand indicates considerable variation in the non-epidemic period also and it may show in the last two weeks of January an increase in other infectious diseases prior to the outbreak of the epidemic proper: this is attributed to the re-assembly of schools. During a non-influenzal period the weekly attack rate of an influenza-like disease did not, however, exceed 5%. The curve for kindergarten children shows still greater fluctuations, due no doubt to the relatively small number of the subjects under observation.

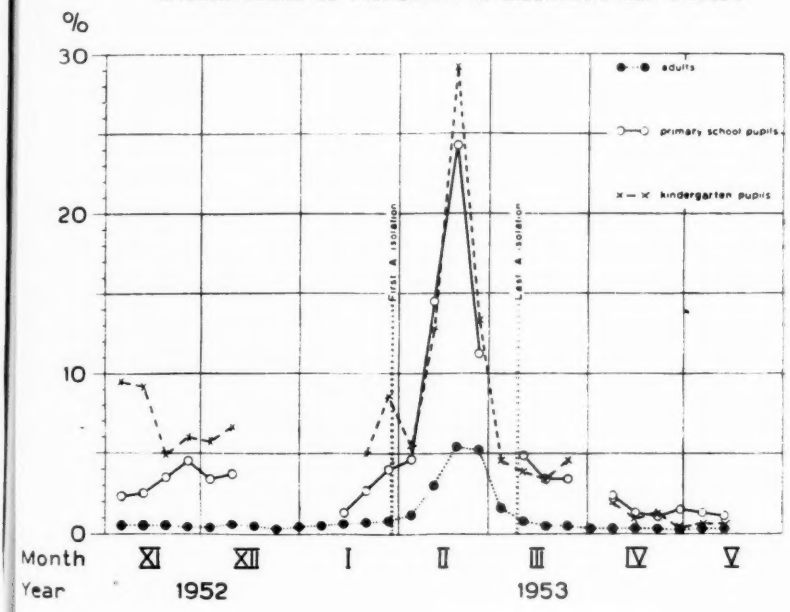
[1] The writer's thanks are due to Dr. U. Muroma, First Medical Officer of Primary Schools, for information received.

In the non-influenzal period the weekly incidence does not exceed 10%.

During the time when influenza virus is found in throatwashings the figures showing the incidence of the disease rise exceedingly sharply and all the curves reach their maximum simultaneously in the third week of February. For adults the peak point is 5.5%, for primary school pupils 24.3 and for kindergarten children 29.2%. It can be estimated from the series of curves, though not very accurately, how large a proportion of each age group has contracted influenza during the epidemic. The estimate is based on the sum total of the successive percentages obtained from each curve during the epidemic period. From each of these percentages has been deducted the percentage representing the estimated normal level, 0.5% for adults, 4% for primary school children and 6% for kindergarten pupils. The total incidence of the disease thus arrived at is 14.2% for adults and 42.5 and 37.4% for primary school pupils and kindergarten children respectively. The estimate thus made can hardly be considered too high. The estimate also makes apparent that the attack rate among primary

GRAPH 1

ATTACK RATES OF INFLUENZA IN DIFFERENT AGE GROUPS



school pupils and kindergarten children has been nearly three times that of adults.

To summarise the foregoing: the influenza epidemic in Helsinki begins to reach its peak and subsides within six weeks. Because of the great evenness shown by it during the non-epidemic period the curve for adults seems to be the most reliable one.

Pneumonia. — During the non-epidemic period pneumonia in adults under observation accounted for an average of 3.6 cases per week before the epidemic and for 4.5 cases per week after it. During the epidemic for five weeks the weekly figures were 4, 24, 10, 14 and 7, their average being 11.8. The high total for the second week may be caused by psychological reasons and only knowledge of the nature of influenza excludes incorrect diagnoses. The influenza epidemic thus seems only to have doubled the number of pneumonias.

Mortality Attributed to Influenza. — Death certificates of persons dying in Helsinki in the course of Spring 1953 mentioned influenza as the immediate cause of death or as a contributory factor in 32 cases. The following table shows the weekly grouping of the cases.

TABLE 1

MORTALITY IN WHICH INFLUENZA IS REGARDED AS THE IMMEDIATE CAUSE OR CONTRIBUTORY FACTOR

Period	February				March			
	1—7	8—14	15—21	22—28	1—7	8—14	15—21	Later
Number	1	1	6	10	6	5	3	1

The table reveals that the highest influenza mortality rate seems to have coincided with the week following the maximum incidence of the disease. Doubtless there are some incorrect diagnoses among the cases but the grouping, illustrative of the general course of the epidemic, probably supports the finding that influenza really has been either the cause of or a contributory factor in the death if we can exclude psychological factors. The average age of persons dying from influenza is 77 years if a 21 year old patient is disregarded with influenza mentioned as the cause of death.

Laboratory Investigations. — The Virus Department of the State Serum Institute (WHO Influenza Centre) has made attempts since

the beginning of 1952 to isolate influenza viruses from garglings collected from suspected cases of influenza. The samples have been recovered mainly from the staff of the public transport company and the railways, post office employees and school children.

Methods. — The patient has been allowed to gargle with about 10 ml. of bacteriological broth. The gargling is then stored frozen at —25° C if it is not possible to examine it immediately. 0.2 ml of the sample, with 500 μg of streptomycin and 500 units of penicillin per ml added, is infected into the amniotic sacs of five eggs incubated at + 38° C for twelve days'. After three days' incubation at + 36.0° C the amniotic and allantoic fluids are collected and their hemagglutinative ability examined against 0.5% of hen and human red cells. The agglutination tests were performed parallelly at room temperature and in a refrigerator. When the hemagglutinating titre was sufficiently high the anti-influenza A (PR8), anti-influenza A' (A/Finland/1/51) and anti-influenza B (Lee) hen sera were used for type determination. The final type determination was kindly performed by Dr. Alick Isaacs (WHO Influenza Centre, London) to whom the writer is greatly indebted.

119 throat-washings were examined in the course of 1952, all of them giving a negative result. The number of investigations performed at the beginning of 1953 and the number of virus strains found in them become clear from Table 2. The date of the first positive sample was January 29 and of the last March 9.

TABLE 2

NUMBER OF VIRUS STRAINS ISOLATED DURING EPIDEMIC

Period	January		February				March			
	1—24	25—31	1—7	8—14	15—21	22—28	1—7	8—14	15—	T
Samples	7	5	13	23	7	8	4	3	11	81
Positive	—	2	3	10	6	5	—	2	—	28
Negative	7	3	10	13	1	3	4	1	11	53

Not a single positive sample was found after March. Table 2 shows that 24, i.e. 47%, of the 51 samples examined during February were positive.

Type Determinations. — According to the preliminary determinations performed all 28 isolated strains belonged to the A' group of influenza viruses. Dr. Alick Isaacs performed the more accurate type determination of 22 isolated strains and ascertained that 18 of the strains belong to the Scandinavian sub-type and 4 to the Liverpool sub-type. Two virus types, serologically distinguishable, have thus been involved in the epidemic in question although it was chiefly caused by the Scandinavian variant.

The results of the serological investigations will be published later.

SUMMARY AND DISCUSSION

The paper deals with the influenza epidemic of Spring 1953 in Helsinki. The curves illustrating the absence from work, school or kindergarten caused by an influenza-like disease from November 1952 to June 1953 are introduced by age groups. The section of population under observation comprised nearly 10% of the total population of Helsinki. The curves show that the incidence of influenza among school children and kindergarten pupils was almost three times that of adults. The number of pneumonias seems to be about double that of the non-epidemic period. Influenza has been estimated, on the basis of death certificate data, to have caused or have contributed to the death of 32 persons. The average age of people dying from influenza has been 77 years.

The laboratory tests performed proved that 24, i.e. 47%, of the 51 garglings recovered during February were positive. The 28 virus strains isolated during the epidemic belonged to the A' group. Of the 22 strains examined more closely 18 were of the Scandinavian and 4 of the Liverpool sub-type.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

INFLUENCE OF CORTISONE ON MITOSIS

II

EFFECTS OF SIMULTANEOUSLY APPLIED CORTISONE AND COLCHICINE

by

A. ISOTALO and H. TEIR

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Cortisone has been proved to have an antimitotic effect upon the cells of the epidermis of rats (3, 5) and of mice (2). This substance has not a similar effect upon the outer orbital gland of the rat (5). Since a combination of cortisone and colchicine was used as a method of studying the effect of cortisone on the mitosis of the epidermis (1, 3) we thought it interesting to investigate whether the difference that we observed in the effect of cortisone on mitosis in different tissues could be still further confirmed in this way.

METHODS

The material investigated consisted of the outer orbital gland and the epidermis of 58 young rats of the Wistar strain. Otherwise the method was the same as that used in our previous cortisone investigations (5).

RESULTS

The investigation comprised three different series of experiments.

Experiment No. 1. — 0.5 mg of cortisone was injected intraperitoneally and 0.05 mg of colchicine subcutaneously into 1-month-

old rats. The time of influence was 12 hours for colchicine and 5 hours for cortisone. The following mitotic values were obtained:

	Outer Orbital Gland Individual Mit. Ratio	Epidermis Individual Mit. Ratio
Cortisone 5 hours	24, 26,	—, 1
» 12 »	10, 36, 12	3, 4, 3,
Colchicine 5 »	39, 652, 108	66, 45, 23
» 12 »	330, 34	30, 48
Cortisone + 5 »	15, 2, 88, 50	16, 11, 28, 34
colchicine 12 »	8, 174, 102, 85	17, 10, —, —
Saline sol. 5 »	3, 8, 12	13, 9, 8
» » 12 »	14, 20, 19	8, 7, 7

With cortisone alone no certain antimitotic effect was obtained in the outer orbital gland, whereas such an effect was observed in the epidermis. Owing to the abundance of metaphaseblocked mitoses, high mitotic values were obtained with colchicine in both these organs. Simultaneous application of cortisone and colchicine gave lower mitotic values than colchicine alone.

Experiment No. 2. — In order further to control the result of the first experimental series 5 mg of cortisone were injected into 4 rats, 0.05 mg of colchicine into 4 rats and equal quantities of both these substances into 9 1-month-old rats. In all the experiments the time of influence was 5 hours. The weight of the animals varied between 56 and 62 g. The following mitotic values were obtained:

	Outer Orbital Gland		Epidermis	
	Individual Mit. Ratio	Mean Value	Individual Mit. Ratio	Mean Value
Cortisone	0, 0, 2, 2	1 ± 0.6	0, 1, 2, 0	1 ± 0.5
Colchicine	29, 34, 32, 17	28 ± 3.8	26, 13, 2, 16	14 ± 4.9
Cortisone +	11, 87, 8, 8, 44		2, 1, 0, 4, 6	
Colchicine	0, 2, 10, 5	19 ± 9.5	5, 4, 2, 3	3 ± 0.6

Cortisone again reduced the colchicine-influenced mitotic ratio both in the skin and in the outer orbital gland.

Experiment No. 3. — Since the outer orbital gland is one of the stabile organs in which mitoses occur almost exclusively during the growth period, and since the skin belongs to the labile organs in which a continuous physiological wastage and replacement of cells takes place, we made yet another experiment with rats aged 3 months and 18 days. Since an optimal colchicine effect is obtained in adult animals after about 1 day (4) this time of influence was used for the colchicine, of which 0.25 mg was injected subcutaneously. The cortisone dose was 10 mg and was given intra-abdominally. The time of influence was 5 hours. The weight of the experimental animals varied between 112 and 190 g. The following mitotic values were obtained:

	Outer Orbital Gland		Epidermis	
	Individual Mit. Ratio	Mean Value	Individual Mit. Ratio	Mean Value
Cortisone	3, 0, 4, 4	3 ± 0.9	3, 1, 3, 1	2 ± 0.6
Colchicine	16, 136, 188, 106	112 ± 36.1	91, 137, 77	102 ± 18.1
Cortisone + colchicine	32, 33, 16, 24, 21	25 ± 3.2	36, 18, 27, 25, 13	24 ± 3.9
Controls	0, 2, 2, 2	2 ± 0.5	0, 1, 5, 6	3 ± 1.5

The result was again the same. The rats receiving both colchicine and cortisone displayed clearly reduced mitotic values as compared with the rats that received colchicine only.

DISCUSSION

The experiments show that cortisone has an antimitotic effect upon the epidermis, as has been demonstrated earlier (1, 2, 5). As was also the case in our previous investigation (5) we could not establish any such effect of cortisone on the outer orbital gland. With colchicine typical colchicine mitoses were obtained both in the epidermis and in the outer orbital gland. The metaphase-blocked mitoses consirably increased the tmiotic ratio in the animals. High mitotic values were obtained in the outer orbital gland with colchicine even in animals over 3 months old. The mitotic ratio was often so high that it may be questioned whether

colchicine had not here a stimulating effect such as was earlier found to occur in stabile organs of adult animals (3, 6).

On simultaneous application of cortisone and colchicine the same result was obtained both in the orbital gland and in the epidermis in all experiments. Cortisone decreased the colchicine mitotic ratio. In view of the antimitotic effect of cortisone in the epidermis the effect in this organ is quite understantable. It is more difficult to explain the effect in the outer orbital gland; even large doses of cortisone by themselves have no antimitotic effect on this organ (5). It is possible that cortisone when applied together with colchicine influences mitosis even in organs in which cortisone alone does not effects mitosis.

The effect of cortisone on the colchicine mitotic ratio should be considered in experiments in which these two substances are simultaneously given to experimental animals.

SUMMARY

Cortisone has an antimitotic effect in the epidermis but not in the outer orbital gland of the rat. On simultaneous application of cortisone and colchicine, cortisone decreased the colchicine mitotic ratio both in the epidermis and in the outer orbital gland.

REFERENCES

1. Bullough, W. S.: J. Endocrinol. 1952:*8*:265.
2. Green, H. N., and Ghadially, F. N.: British Med. J. 1951:*1*:496.
3. Studer, A., and Frey, J. R.: Dermatologica 1952:*104*:1.
4. Teir, H.: Acta Pathol. et Microbiol. Scand. 1944:Suppl.*54*.
5. Teir, H., and Isotalo, A.: Ann. Exper. Med. & Biol. Fenn. 1953:*31*:171.
6. Teir, H., and Ravanti, K.: Exptl. Cell Research 1953. In press.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

DISTRIBUTION OF INTRAPERITONEALLY INJECTED RAT TISSUE SUSPENSIONS LABELLED WITH P^{32} [1]

by

T. KUUSI, A. ISOTALO, and H. TEIR

(Received for publication August 22, 1953)

When a tissue suspension derived from the orbital gland of the rat is injected intraperitoneally, stimulation of the mitotic activity of homologous tissue can be observed (7). The stimulus in question is probably not a protein since its effect is not abolished by heat (8). Nucleic acids which, as regards structure, could be tissue specific agents, (1), may act as stimuli. Should this be the case, then the tissue specific action of the suspension might perhaps be proved by labelling the suspension with radioactive phosphorus, P^{32}. The labelled compounds of the orbital gland suspension injected might be transferred specifically to the orbital gland of the acceptor animal.

MATERIAL AND METHODS

In the experiments, white rats about 1-month-old were used, into which 5—860 μC P^{32} in an inorganic form (Na_2HPO_4) were injected intraperitoneally. The radioactivity of the specimens was determined as counts/min. per mg. fresh weight (5). The uptake by the following organs was determined: liver, thymus, extraorbital gland, forestomach, glandular stomach, muscle, skin, small intestine, infraorbital gland, parotid gland and Harder's gland. The time of incorporation of the inorganic P^{32} varied between 1 hour and 2 days, that of the labelled tissue suspension between 1 hour and 5 days.

[1] An investigation aided by a grant from the Sigrid Jusélius Foundation, Helsinki, and an institutional grant from the Damon Runyon Memorial Fund, New York.

RESULTS

Incorporation of Inorganic P^{32}

Experiment No. 1. — The quantity of P^{32} was 5 μC and the time of incorporation 1,24 and 48 hours. The result appears from fig. 1,

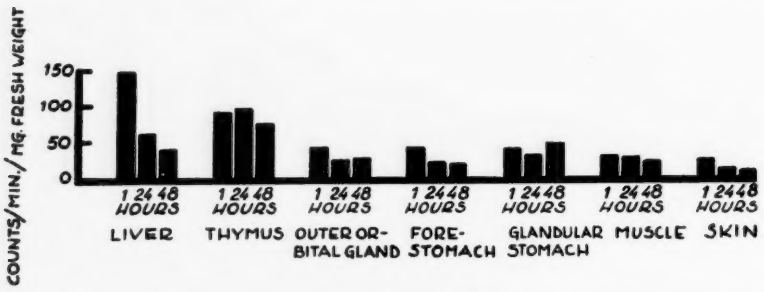

Fig. 1. — Uptake by different organs after an intraperitoneal injection of 5 μC inorganic P^{32}. Experiment no. 1.

and tallies rather well with the results of earlier investigations (2, 3). The uptake by the orbital gland has, however, scarcely been investigated before. From the results of this experiment the radioactivity of the suspensions in the following experiments may be estimated. Further this distribution forms the basis with which the results of the following experiments should be compared when studying a possible tissue specificity.

Incorporation of Tissue Suspension Labelled with P^{32}

Experiment No. 2. — 50 μC P^{32} were injected into each of two rats. The rats were decapitated after one hour and tissue suspensions were made of the liver, the thymus and the orbital gland. The dosis per acceptor rat was about 315,000 c/min. in the liver suspension, about 38,600 c/min. in the thymus suspension and about 26,500 c/min. in the orbital gland suspension. Each suspension was injected into three rats, which were killed 1,24 and 48 hours after injection. Fig. 2 shows the distribution of the labelled compounds of the orbital gland suspension. When comparing the result with the distribution in experiment no. 1, it appears that it is more or less the same, with the exception of the peaks after 1 hour in the forestomach and the glandular stomach. On the other hand the uptake by the orbital gland, when, for instance, compared with that of the

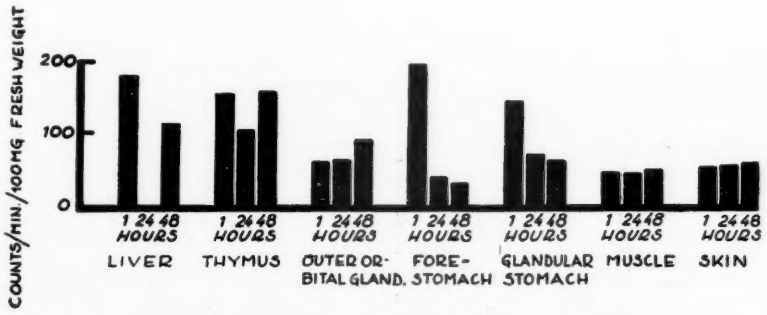

Fig. 2. — Uptake by different organs after an intraperitoneal injection of P^{32}-labelled orbital gland suspension. Experiment no. 2.

liver and the thymus, is about the same as when inorganic P^{32} is used.

Neither did similar experiments with liver and thymus suspension indicate that the labelled compounds of the liver suspension had been transferred specifically to the liver, or that of the thymus suspension specifically to the thymus.

Experiment No. 3. — Since, in the orbital gland suspension series of the previous experiment, the uptake by the orbital gland showed a tendency to increase, it was considered necessary to perform yet another experiment allowing a longer time of incorporation for the radioactive suspension. Simultaneously the time of incorporation of the inorganic P^{32} in the primary rats was prolonged. The total radioactivity in the suspension undoubtedly decreases in consequence, but the P^{32} has probably to a large extent been metabolized into an organic form.

Each of two rats received an injection of 350 μC P^{32}. The rats were decapitated after 24 hours. Suspensions of the liver, the thymus and the orbital gland were made in the usual way. The dosis per acceptor rat was about 95,200 c/min. in the liver suspension, about 79,100 c/min. in the thymus suspension and about 26,500 c/min. in the orbital gland suspension. Each suspension was injected into 4 rats, which were killed 1, 2, 3 and 5 days after injection. The effect to the orbital gland suspension appears from fig. 3.

When comparing the result with the distribution in experiment no. 1 it is seen that the uptake by the liver and of the thymus,

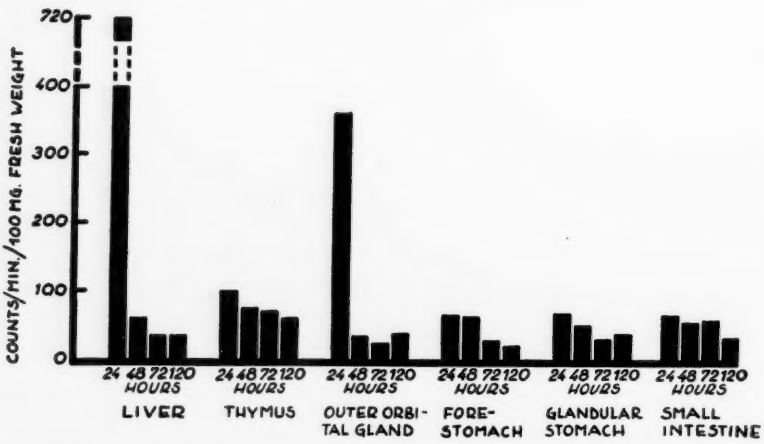

Fig. 3. — Uptake by different organs after an intraperitoneal injection of P^{32}-labelled orbital gland suspension. Experiment no. 3.

which in experiment no. 1 considerably exceed the rest, are less conspicuous in this experiment, even though the initial value for the liver is very high. In the orbital gland also a remarkably high value can be observed after 1 day, after which the radioactivity rapidly decreases.

There is, however, a similar peak in the orbital gland 1 day after injection of thymus suspension, the uptake being even higher than the corresponding values for the liver and for the thymus

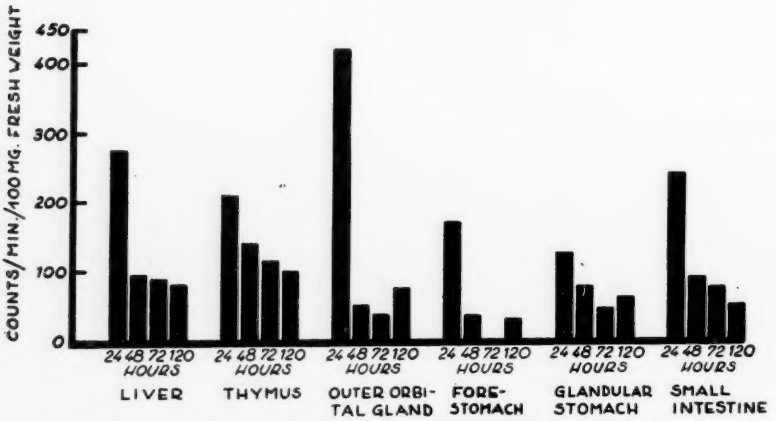

Fig. 4. — Uptake by different organs after an intraperitoneal injection of P^{32}-labelled thymus suspension. Experiment no. 3.

(fig. 4). The peak in the diagram 3 cannot therefore be considered a reliable sign of specific incorporation of the labelled compounds of the orbital gland suspension into the orbital gland, since the orbital gland to an even greater extent incorporates that of the thymus suspension.

Experiment No. 4. — In order to establish whether the peak in the orbital gland series, observed in the previous experiment, is reproducible, another experiment was performed, in which the uptake by the infraorbital, the parotid and Harder's gland also were measured. The infraorbital gland is homologous with the extraorbital.

Into each of 4 rats 860 μC P^{32} were injected. The rats were decapitated after 24 hours and a suspension was made of their orbital glands. The dosis per acceptor rat was about 58,600 c/min. The radioactive suspension was injected into 8 rats. The time of incorporation was 1, 12, 24 and 48 hours. There were two rats in each group. The distribution of the labelled compounds appears from fig. 5.

Comparison of the result with the distribution in experiment no. 1 shows that, in the two diagrams, the uptake by the orbital gland is to the same extent lower than that of the liver and the thymus. The peak in the collection of labelled compounds by the orbital gland seen in experiment no. 3 after 1 day, did not occur in this experiment. The uptake of the orbital gland was very much

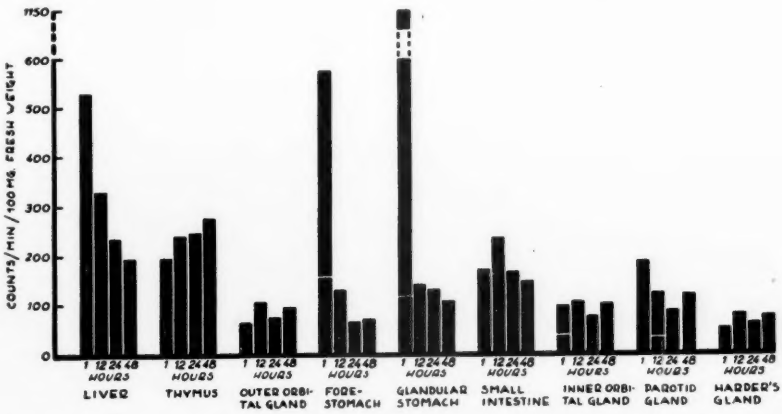

Fig. 5. — Uptake by different organs after an intraperitoneal injection of P^{32}-labelled orbital gland suspension. Experiment no. 4.

the same degree as that of the infraorbital, the parotid and the Harder's glands investigated for the sake of comparison. It is therefore obvious that no specific transfer of the labelled orbital gland suspension to the orbital gland of secondary rats could be proved with certainty in these experiments.

CONCLUSIONS

Specificity. — It appears from our experiments that the radioactive material of a tissue suspension labelled with P^{32} is obviously not transferred specifically to homologous tissue. The distribution of the radioactivity in these experiments is more or less the same as after injection of the inorganic P^{32}. Almost similar results were obtained with suspensions of liver, thymus and orbital gland in this respect.

When interpreting this result it should be born in mind that the experimental conditions are not quite the same as those in the experiments on stimulation of mitosis. Since we tried to obtain as high a final activity of the organs as possible, we used considerably higher concentrations of tissue suspension than those optimal for stimulation of mitosis (7, 8).

These experiments with P^{32} primarily provide a picture of incorporation into the orbital gland of inorganic and more or less organically bound P^{32}. We have not investigated the distribution of P^{32} in the labelled tissue suspensions between the different fractions, nor have the different fractions been separately injected. It is nor certain whether the phosphorus compounds are responsible for the stimulation of mitosis. That an accumulation of the labelled tissue suspension in a homologous organ could not with certainty be discerned in these experiments might indicate that the agent in question cannot be labelled with phosphorus. We cannot be certain, however, that such a conclusion is correct, because the chemical nature of the mitosis stimulating agent is still unknown.

It is possible that, by using another technique, for instance, by fractionating the tissue suspensions, other results might be obtained. So, with intravenously injected chromatin fractions from the liver of the rat labelled with P^{32} Marshak and Walker got a distinctly greater uptake in the liver nuclei in relation to the uptake of inorganic phosphate (6). Marshak and Walker's observation that

labelled fractions from the rat liver were much more effective than fractions from the rabbit liver labelled in the same way, argues in favour of the existence of a species specificity.

Observations on Phosphorus Metabolism. — The experiment does not provide much information on the phosphorus metabolism of the orbital gland. However, some conclusions might be drawn on the basis of the total incorporation. It may, for instance, be observed that the uptake in the orbital gland is almost independent of the initial concentration of labelled compounds in the injected suspension. This would indicate that the gland had reached some kind of saturation with regard to P^{32}.

It is remarkable that, in experiment no. 3, an uptake maximum of the orbital gland was observed after 1 day and this maximum was considerably higher than the uptake in later stages. The uptake maximum of the orbital gland is not equally apparent in the other diagrams, perhaps because no determination coincided with the time of the maximum. This change of the uptake mostly resembles the corresponding change for the liver. It might therefore be concluded that the P-metabolism of the orbital gland is of the same type as that of the liver but that it differs from that of the thymus. In the liver a rapidly rising maximum can be observed and thereafter a rapid descent, which shows that liver incorporates and likewise gets rid of P^{32} quickly, while in the thymus the uptake generally shows a slow rise and thereafter a slow descent; consequently both P^{32} incorporation and elimination by thymus are slow. The orbital gland, therefore, is of the type with rapid P-metabolism. This agrees well with the cytological observation that the cytoplasmic basophilia and the ribonucleic acid content of the orbital gland is remarkably high (4); metabolically ribonucleic acid is generally much more active than desoxyribonucleic acid. Quantitatively the P^{32} incorporation in the orbital gland is smaller than in the liver and the thymus.

SUMMARY

P^{32}-labelled tissue suspensions of liver, thymus and orbital gland were injected intraperitoneally. Specific incorporation of the suspension into homologous tissues could not be established. Some features about the phosphorus metabolism of the orbital gland were observed.

REFERENCES

1. CHARGAFF, E.: Experientia 1950:*6:*201.
2. HEVESY, G.: J. Chem. Soc. 1939:1213.
3. HEVESY, G.: Radioactive Indicators. New York 1948.
4. KUUSI, T., and H. TEIR: 1953; unpublished.
5. LAMBERG, B.-A.: Acta Med. Scand. 1953:Suppl. 279.
6. MARSHAK, A., and WALKER, A.: Am. J. Phys. 1945:*143:*235.
7. TEIR, H.: Acta Path. et Microbiol. Scand. 1952:*30:*158.
8. TEIR, H.: Growth 1952:*16:*85.

AUS DER I UND DER II UNIV. FRAUENKLINIK ZU HELSINKI

UNTERSUCHUNGEN ÜBER DEN GEHALT DES TUMORGEWEBES AN FREIER CHROMOTROPER SUBSTANZ UND MASTZELLEN BEI VERSCHIEDENEN FORMEN VON CYSTADENOMA SEROSUM OVARII

von

C. VON NUMERS und P. FORTELIUS

(Am 3. 9. 1953 bei der Schriftleitung eingegangen)

Die ungeformte, strukturlose Grundsubstanz des Mesenchyms ist, nachdem ihre grosse Bedeutung bei verschiedenen sowohl physiologischen als auch pathologischen Geschehen entdeckt und anerkannt worden war, Gegenstand einer eifrigen Forschungsarbeit gewesen. Der schleimartige, »mucinöse» Charakter dieser Matrix, seit altersher bekannt von der Wharton'schen Sulze des Nabelstrangs, wurde von Virchow (9) und Rollet (8) auch im Bindegewebe des erwachsenen Organismus nachgewiesen. Wie man nunmehr weiss, besteht diese visköse, amorphe Grundsubstanz aus hochmolekulären Polysacchariden in Verbindung mit Proteinen, von welchen die ersteren dank der für sie spezifischen histochemischen Reaktionen ein geeignetes und viel behandeltes Untersuchungsobjekt darstellen. Von solchen Reaktionen hat neben der von McManus (6) und Hotchkiss (3) eingeführten Methode mit Perjodsäure und Schiffs Reagens die mit gewissen basischen Farbstoffen erhaltene Metachromasie Anwendung gefunden. Die auf Lisons (5) umfangreichen Untersuchungen basierende Ansicht, wonach bei Färbung z.B. mit Toluidinblau nur die schwefelhaltigen Polysaccharide metachromatische Reaktion ergeben sollten, hat sich später als unrichtig erwiesen. So hat u.a. Asboe-

Hansen (1) konstatiert, dass sich auch die schwefelfreie Hyaluronsäure metachromatisch färbt. In einer langen Serie von Untersuchungen hat Sylvén nachgewiesen, dass der Gehalt an Polysacchariden im Bindegewebe erhöht ist bei Epithelregeneration, in wachsenden mesenchymalen Tumoren sowie im Stroma bei rasch wachsenden epithelialen Tumoren. Mit aller Wahrscheinlichkeit sind die quantitativen Variationen der metachromatischen Reaktion weitgehend vom verschiedenen Polymerisationsgrad der Polysaccharide abhängig.

Die von Holmgren und Wilander (2) vertretene Ansicht, dass die stark metachromatischen Granula der Mastzellen aus Heparin bestünden, ist in gewissem Masse durch die Untersuchungen von Asboe-Hansen modifiziert worden. Nach diesem Forscher bilden die Mastzellen unter hormonaler Einwirkung Hyaluronsäure, eventuell über ein heparinähnliches Vorstadium. Asboe-Hansen hat in Übereinstimmung mit früheren Forschern eine Anhäufung von Mastzellen in oder in der Umgebung von Gewebspartien, in denen erhöhter Gehalt an freier metachromatischer Substanz nachgewiesen werden kann, beobachtet.

Ein geeignetes Objekt für die Untersuchung von freier chromotroper Substanz und Mastzellen bilden im gleichen Organ vorkommende, morphologisch gleichgeartete pathologische Prozesse, die sich durch verschiedenen Grad der Zuwachspotenz voneinander unterscheiden. In einer solchen Serie sollte gemäss der obigen Ausführungen parallel mit steigender Wachstumsgeschwindigkeit eine Vermehrung der metachromatischen Substanz bezw. erhöhte Anzahl der Mastzellen zu erwarten sein. Eine hieraus gewonnene Erfahrung könnte unter Umständen sogar in praktisch-diagnostischer Hinsicht von Bedeutung werden bei der Beurteilung des Malignitätsgrads des Tumors in zweifelhaften Fällen. Bezüglich der weiblichen Milchdrüse ist eine derartige Serienuntersuchung kürzlich durchgeführt worden (7) mit im obenerwähnten Sinne im grossen und ganzen positivem, obwohl nicht durchgehend eindeutigem Resultat. In der Absicht, die diesbezüglichen Forschungen weiterzuführen, haben wir nunmehr als Unter suchungsobjekt die verschiedenen Formen von Cystadenoma serosum ovarii gewählt. Das Tumormaterial stammt aus der I und II Universitätsfrauenklinik zu Helsinki und besteht aus in Formalin fixierten sowie in Paraffin eingeschlossenen Probestücken. Obwohl

die Formalinfixierung nicht als ideale Methode bei Untersuchungen von der in Frage stehenden Art angesehen werden kann, so lässt sie sich doch, wie aus den Untersuchungen von Sylvén hervorgeht, erfolgreich verwenden, sofern die Fixierungszeit nicht zu lange gewesen ist. In unserem Material überstieg dieselbe nicht 24 Stunden. Das Präparat wurde in einer 0.5 %igen Lösung von Toluidinblau in destilliertem Wasser gefärbt, worauf eine rasche Entwässerung in 96 %igem Alkohol durchgeführt wurde.

Insgesamt sind 31 Tumoren auf das Vorkommen von freier metachromatischer Substanz und Mastzellen im Stromagewebe untersucht worden. Daneben wurden ausserdem Beobachtungen über eventuell im Epithel vorkommende Mucopolysaccharide gemacht. Sämtliche Tumoren wiesen die für das seröse Zystadenom charakteristischen morphologischen Sonderzüge auf: meistens uni- oder paucilokuläre Zystenbildungen mit serösem, nicht fadenziehendem Inhalt sowie ein in typischen Fällen relativ niedriges, oft kubisches Epithel mit zentral gelegenen Kernen, in welchem Flimmerzellen in höherer oder minderer Anzahl vorkommen. Das untersuchte Material wurde in vier Gruppen eingeteilt, von denen die erste das einfache, nicht papillenbildende Zystadenom (Cystadenoma serosum simplex) umfasst, die zweite wiederum das papillenbildende, aber sowohl in histologischer als auch klinischer Hinsicht unzweideutig gutartige Zystadenom. Zur dritten Gruppe wurden in Übereinstimmung mit den von Kottmeier (4) vorgeschlagenen Einteilungsprinzipien solche papillenbildende Zystadenome gezählt, deren Epithel deutlich vermehrte Proliferation und Atypie aufwies, während Zeichen von infiltrativem Wachstum fehlen. Wie Kottmeier gezeigt hat, nehmen diese suspekten, aber nicht mit Sicherheit malignen Zystadenomen auch in klinischer Hinsicht eine Mittelstellung ein. Es hat sich erwiesen, dass sie prognostisch entschieden günstiger sind als die unzweifelhaft karzinomatösen Formen mit infiltrativ wachsendem Epithel, welche die vierte Gruppe unseres Untersuchungsmaterials ausmachen.

Die Verteilung des Materials auf die verschiedenen Gruppen geht hervor aus Tabelle 1, worin auch das Resultat der Untersuchung in den Hauptzügen wiedergegeben ist. Obwohl einer nur auf subjektiver Beurteilung beruhenden quantitativen Schätzung nur bedingter Wert zugesprochen werden kann, dürften doch die

TABELLE

Gruppe	Zahl d. Fälle	Zystenwand							
		Freie chromotr. Subst.				Mastzellen			
		—	(+)	+	++	—	(+)	+	++
I. Cystaden. Seros. Simplex	8	5	1	2	0	0	3	4	1
II. Cystad. Seros. papillif.	8	5	1	0	1	0	3	3	1
III. » » » carcin.?	7	2	3	1	0	0	6	0	0
IV. » » » carcin.	8	0	3	2	0	0	3	1	1

Verhältnisse in grossen Zügen zu sehen sein. In der Tabelle wird spärliches Vorkommen von freier metachromatischer Substanz bezw. Mastzellen mit (+) bezeichnet, mässiges Vorkommen mit + sowie reichlicheres mit ++.

Wie a priori zu erwarten war, ist die aus derbem, faserreichem und zellarmem Bindegewebe bestehende eigentliche Zystenwand im allgemeinen arm an Mucopolysacchariden und ist auch nicht nachweislich durch die Wachstumsgeschwindigkeit des Tumors beeinflusst. In denjenigen Fällen, in denen positive Färbung konstatiert wurde, war dieselbe auf zunächst unter dem Epithel gelegene Gewebspartien lokalisiert. Nur in der vierten Gruppe lässt sich eine Verschiebung zu reichlicherer Metachromasie beobachten, die vielleicht durch die stellenweise vorkommende Infiltration von Karzinomgewebe bedingt ist. Bezüglich der Frequenz der Mastzellen lassen sich keine deutlichen Unterschiede zwischen den verschiedenen Gruppen nachweisen.

Das Bindegewebe der Papillen erwies sich, wie auch zu vermuten war, als reicher sowohl an freier chromotroper Substanz als auch an Mastzellen. Hier lässt sich auch betreffs der beiden letzten Gruppen eine deutliche Vermehrung beider Komponenten im Vergleich zu dem gutartigen papillären Zystadenom feststellen. In Übereinstimmung mit früher gemachten Erfahrungen in Bezug auf andere Tumoren sind also auch beim serösen Zystadenom reichlichere Epithelproliferation und grössere Wachstumsgeschwindigkeit begleitet von zunehmenden Mengen freier chromotroper Substanz und erhöhter Anzahl der Mastzellen im Stroma.

Hier müssen einige nähere Erklärungen bezüglich gewisser histologischer Beobachtungen, die aus der Tabelle nicht ersichtlich sind, beigefügt werden. Da in jedem einzelnen Falle beträchtliche

1

Papillenbindegewebe								Epithel				
Freie chromotr. Subst.				Mastzellen								
—	(+)	+	++	—	(+)	+	++	—	(+)	+	++	
—	—	—	—	—	—	—	—	5	1	2	0	
1	3	2	2	0	3	2	3	2	3	3	0	Zystenwand fehlt in 1 Falle
0	2	2	3	0	0	3	4	0	0	3	4	» » » »
0	1	2	5	0	0	5	3	0	3	4	1	» » » 3 Fällen

Variationen bezüglich des Gehalts des Tumorgewebes an Mucopolysacchariden und Mastzellen vorkommen, ist meistens bei der Klassifizierung des Tumors dasjenige Gebiet ausschlaggebend gewesen, in welchem die stärkste metachromatische Reaktion wahrgenommen wurde. Die Ungleichmässigkeit, mit der die Toluidinblaureaktion ausfällt, scheint ein charakteristischer Sonderzug für dieselbe zu sein, zum mindesten in dem formalinfixierten Klinikmaterial, das hier benutzt wurde. Gebiete mit negativer und deutlich positiver Reaktion kommen wechselweise nebeneinander vor und weisen keine anderen histologischen Verschiedenheiten auf, die die lokalen Variationen auch nur einigermassen erklären könnten. Da es weniger wahrscheinlich erscheint, dass der Gehalt des Tumorgewebes an Polysacchariden tatsächlich so schroffen lokalem Wechsel unterworfen wäre, dürfte die Ursache wohl in der Launenhaftigkeit der histochemischen Reaktion zu suchen sein. Dieser Umstand trägt selbstverständlich dazu bei, ihre praktische Bedeutung erheblich zu vermindern.

Besondere Aufmerksamkeit haben wir dem amorphen, sehr zellarmen Stroma zugewandt, das so oft die Papillen des serösen Zystadenoms auszeichnet. Zum überwiegenden Teil hat dieses hydropisch aussehende Stromagewebe sich bei der Toluidinblaufärbung negativ verhalten. Nur in Ausnahmefällen haben wir innerhalb kleinerer Gebiete schwache oder mässige metachromatische Reaktion beobachtet. Auf Grund dieser Wahrnehmungen dürfte man annehmen können, dass bei diesem veränderten Papillenstroma keineswegs von einer »schleimigen Entartung» die Rede sein kann.

Schliesslich noch einige Worte zum Verhalten des Tumorepithels. Wie aus der Tabelle hervorgeht, ist in allen vier Gruppen das Vorkommen von metachromatisch gefärbtem Sekret im Epithel nach-

gewiesen worden. In den Fällen, bei denen positiv gefärbte Zellen beobachtet wurden, kamen diese in relativ geringer Menge, unregelmässig zwischen negativ reagierende Zellen eingestreut vor. Das mucopolysaccharidhaltige Sekret erscheint in Form einer apikal in der Zelle gelegenen Vakuole von wechselnder Grösse, deren Inhalt aus staubartig feinen, metachromatisch gefärbten Partikelchen besteht. Stellenweise kann man sehen, dass solche Granula aus der Zelle ausgestossen wurden. Dementsprechend weist auch der flüssige Inhalt des Tumors oft deutliche metachromatische Färbung auf, wie man es öfters am besten an kleinen Zystenräumen beobachten kann, deren Inhalt im Präparat erhalten geblieben ist. Wir können demnach feststellen, dass die Flüssigkeit des serösen Zystadenoms gleich der des pseudomucinösen Mucopolysaccharide enthält, offenbar aber in weit geringerem Masse. Unseren Untersuchungsresultaten gemäss scheint eine Zunahme der Aktivität des Tumorepithels von einer gesteigerten Abgabe des mucopolysaccharidhaltigen Sekrets begleitet zu sein. Nur bezüglich der mit Sicherheit karzinomatösen Tumoren (Gruppe IV) scheint in dieser Hinsicht ein Rückgang einzutreten, der deutlich durch die Entdifferenzierung des Karzinomepithels bedingt ist.

ZUSAMMENFASSUNG

Insgesamt 31 Fälle von Cystadenoma serosum ovarii sind mittelst Toluidinblaufärbung auf das Vorkommen von freier chromotroper Substanz und Mastzellen untersucht worden. Die Verteilung der Fälle auf die verschiedenen Formen dieser Geschwulstart und die Hauptzüge der Untersuchungsresultate sind aus Tabelle 1 ersichtlich. In der eigentlichen Zystenwand waren keine deutlichen Unterschiede zu erzielen. Im Papillengewebe konnte eine die Aktivität des Epithels begleitende Zunahme von freien Polysacchariden sowie eine vermehrte Anzahl von Mastzellen beobachtet werden. Wenigstens in formolfixiertem Material scheint der Ausfall der metachromatischen Reaktion indessen ziemlich launenhaft und daher weniger zuverlässig zu sein. Im Tumorepithel konnten Zellen mit metachromatisch gefärbten Sekretvakuolen festgestellt werden. Der Zysteninhalt des serösen Zystadenoms enthält Mucopolysaccharide, die offenbar von diesen Epithelzellen stammen.

SCHRIFTTUM

1. ASBOE-HANSEN, G.: Om bindevævets mucinøse substanser. Diss. Kopenhagen 1951.
2. HOLMGREN, HJ., u. WILANDER, O.: Zschr. mikr.-anat. Forsch. 1940:*40:*488.
3. HOTCHKISS, R. D.: Arch. Biochem. 1948:*16:*131.
4. KOTTMEIER, H.-L.: Acta Obst. Gyn. Scand. 1952:*31:*313.
5. LISON, L.: Arch. de biol. 1935:*46:*599.
6. MC MANUS, J. F. A.: Nature 1946:*158:*202.
7. v. NUMERS, C.: Ann. med. exper. et biol. Fenniae. 1953:*31:*199.
8. ROLLET: zit. nach ASBOE-HANSEN.
9. VIRCHOW: zit. nach ASBOE-HANSEN.

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EXPERIMENTAL PRODUCTION OF PULMONARY HYALINE MEMBRANES

by

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To judge from the literature, attempts to produce hyaline membranes by introducing constituents of amniotic fluid into the lungs of experimental animals have not been successful (2). In a previous series of experiments, membranoid formations were obtained by intratracheal injection of a mixture of amniotic fluid, hydrochloric acid, and pepsin into rats (1). Morphologically, however, these membranes were not identical with those seen in newborn babies. Further experiments on other animals resulted in the production of membranes resembling those encountered in the lungs of the human newborn.

Method. — The trachea of the animal was exposed under ether or Nembutal anesthesia, and 1—20 ml of a mixture of amniotic fluid, hydrochloric acid, and pepsin were injected intratracheally. The animal died or was killed within 4—45 hours. The whole lungs were fixed in formalin, and the paraffin preparations were stained by hematoxylin-eosin. Frozen sections from part of the lung tissue were stained in scarlet red. The total number of animals studied in this new series was 55, of which 32 were rats, 4 rabbits, 5 newborn guinea-pigs, and 14 kittens.

Results. — In *rats*, injection often resulted in the formation of membranoid structures, which, however, morphologically differed from those seen in newborn babies. The membranes did not prove fatal to the rats, although some of them were in a poor

condition immediately after the injection. Rapid intratracheal injection of more than 5 ml of the mixture brought about a lasting respiratory arrest. — Marked membrane formation could not be effected in the *rabbits*. These animals showed a poor tolerance to large intratracheal injections, death occurring even after the administration of 20 ml of the mixture, when the amniotic fluid content of the lungs was still small. — Young *kittens* tolerated the injections best of all. They received a mixture of amniotic fluid, pepsin, and hydrochloric acid with a *p*H of 6.0—6.5 in injections of 2—20 ml. Large amounts were usually injected in doses of 5 ml. Only 3 kittens died immediately or within an hour of the injection. The others survived the immediate effects of the injection and either died or were killed within 24 hours. In contrast to the response noted in the rats, respiratory arrest caused by the injection was transient in the majority of the kittens. The appended table shows the results obtained with the kittens. In one case membrane formation was abundant and the membranes resembled those encountered in the lungs of newborn infants (Figs. 1—4). In this case the lungs were partly atelectatic, although the alveolar lumina were not so small as in the neonatal human cases in which pulmonary membranes have been present. The pulmonary atelectasis is visible in the appended microphotographs (Fig. 2): the walls are clearly tortuous, as in a typical non-aerated lung. Squamous cells of amniotic fluid were occasionally seen in the membranes, but the constituents of the amniotic fluid were frequently as difficult to recognize as in the hyaline membranes of newborn infants. — In 4/5 of the week-old *guinea-pigs* membranes could be produced, but in none of these were they so abundant as in some kittens. Morphologically, the membranes produced in the guinea-pigs bore a greater resemblance to those encountered in the human newborn than did those produced in the rats, but they were more atypical than the membranes seen in the kittens. The tolerance of the guinea-pigs to intratracheal injections was nearly as good as that of the kittens.

Comment. — These experiments showed that it is possible to produce hyaline membranes in animals by pulmonary injections in which amniotic fluid is an essential constituent. Yet in none of the cases could hyaline membranes be produced without pneumonia being simultaneously present in the same lung. The fact that

TABLE

PRODUCTION OF PULMONARY HYALINE MEMBRANES IN KITTENS BY INTRATRACHEAL INJECTION OF A MIXTURE OF AMNIOTIC FLUID, HYDROCHLORIC ACID, AND PEPSIN

Kitten No	Amount Injected	Survival after Injection (hours)	Histological Pulmonary Finding		
			Membranes	Pneumonia	Hemorrhages
43	2 cc	Killed 5 h	±	+	+
44	8 cc	» 15 h	—	+	—
45	8 cc	» 15 h	—	+	—
46	10 cc	» 45 h	±	+	+
47	15 cc	» 23 h	+	+	—
48	20 cc	» 35 h	+	+	—
49	20 cc	Died 10 h	++	+	—
62	20 cc	Killed 17 h	±	+	—
65	8 cc	» 12 h	±	+	—
66	18 cc	» 10 h	—	+	—
67	5 cc	» 5 h	±	+	—

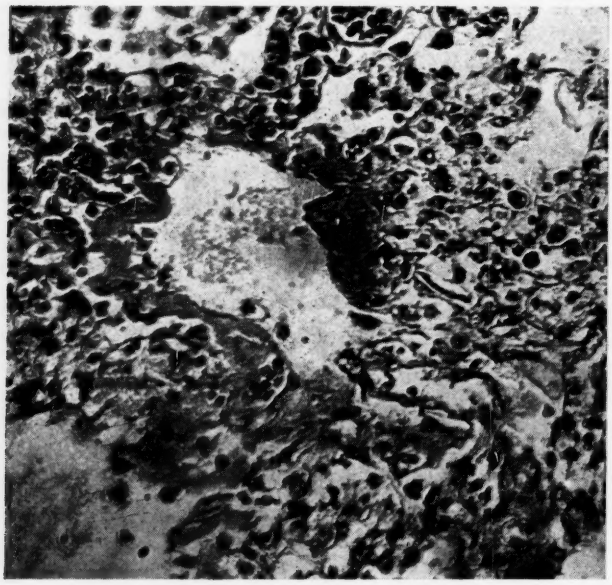

Fig. 1. — Nearly homogenous membranes showing a number of squamous cells. (Kitten 49.)

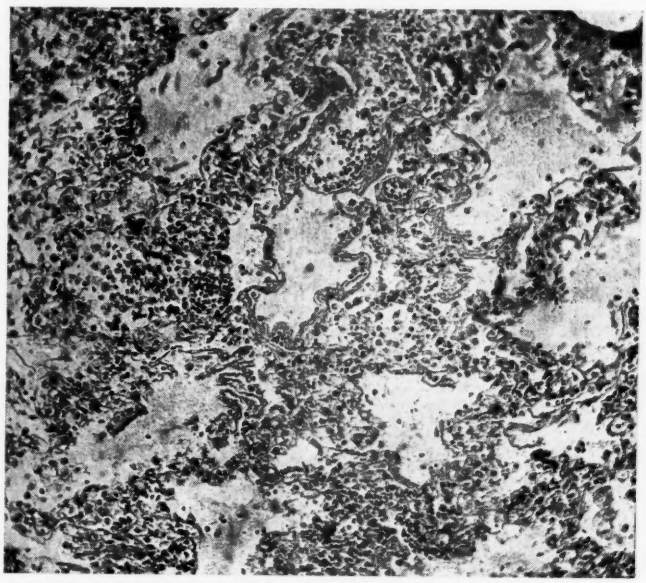

Fig. 2. — Extensive membranous area in an atelectatic lung. The alveolar walls are tortuous. (Kitten 49.)

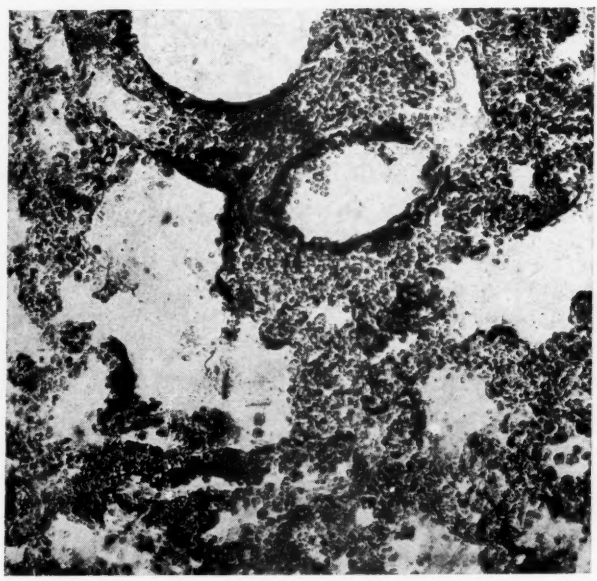

Fig. 3. — The membranes take on an intensive stain with scarlet red, like part of the membranes seen in newborn infants. (Kitten 49.)

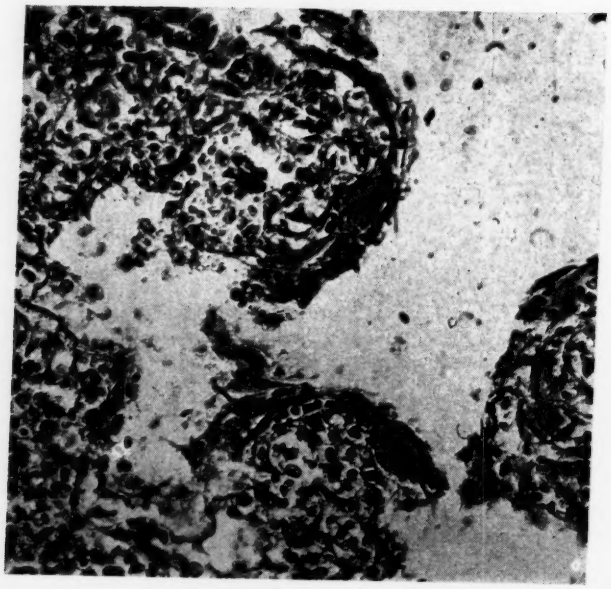

Fig. 4. — The membranes adhere to the walls and are homogenous. (Kitten 49.)

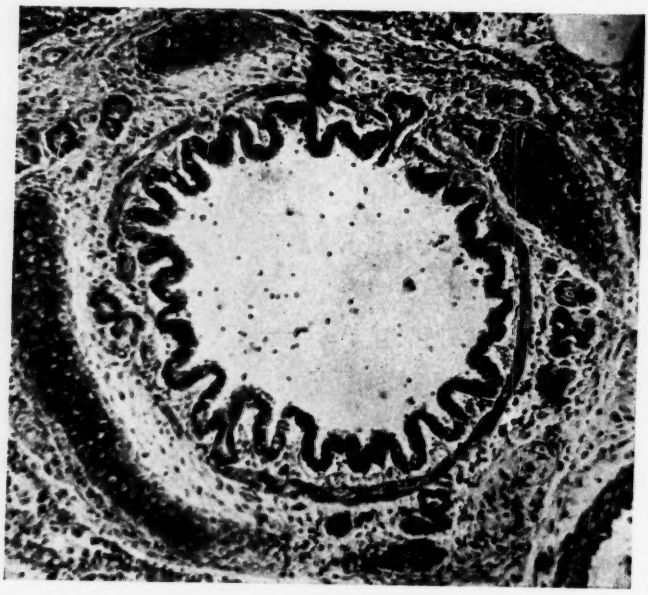

Fig. 5. — Main bronchus with intact epithelium and empty lumen. (Kitten 49.)

membranes also appeared in areas where no leukocytes were found suggests that the pneumonia was not necessarily responsible for the rise of the membranes, but that both were, partially at least, due to the same factor. The experiments gave no definite answer to the question which of the constituents of the amniotic fluid was the principal causative factor in the rise of the membranes nor to the question whether the causative factor was to be found primarily in the amniotic fluid or in the lung. Only one of the animals died spontaneously, all the others being killed; when killed, their condition did not suggest that they would die spontaneously within the next few hours. It was only in the animal which died spontaneously — obviously of a pulmonary cause — that an abundance of membranes were encountered. This hardly justifies a conclusion that the membranes are agonal in origin, for they were present in other cases, too, though not in such a measure. Neither did the experiments prove that pulmonary hyaline membranes are due to the direct action of the injected mixture on the lung tissue or to its arrangement as membranes. Abundant membranes appeared only when large amounts of the mixture were injected. The animal developed severe dyspnea, and there was a transient respiratory arrest as the mixture injected occluded the passages of air. The fact, again, that even smaller amounts injected have given rise to membranes, although in a less measure, suggests that the effect is local. The failure of the experiments with adult animals is probably due to a lack of tolerance to the aspiration of amounts sufficient for the production of membranes, the result being suffocation. The aspiration of large amounts of amniotic fluid seems to be requisite for the appearance of membranes because a considerable proportion of the amniotic fluid is absorbed and only the solid constituents and possibly a part of the protein content are left.

On the basis of previous experiments of this kind the theory has been put forward that one source for neonatal hyaline membranes may be the aspiration of swallowed and vomited amniotic fluid (1). There is some indirect evidence for such a view. The fact that the bronchial epithelium may be normal or almost normal in newborn infants with pulmonary hyaline membranes does not prove that no aspiration of gastric contents has taken place, for in the experiments here reported the epithelium of the main bronchi hardly

exhibited any recognisable changes (Fig. 5). The gastric contents aspirated by a newborn infant do not seem to be absorbed to the same extent as amniotic fluid because they are thicker. There seems to be reason to assume, therefore, that a smaller amount of gastric contents is sufficient for producing membranes in a newborn infant than the amount of amniotic fluid used in the experiments now reported. In any case, it would be rash to draw any final conclusions on the pathogenesis of pulmonary hyaline membranes in the newborn from the results of this series of experiments. Further studies on the subject are therefore required.

SUMMARY

A mixture of amniotic fluid, hydrochloric acid, and pepsin was intratracheally injected into a number of various animals in an attempt to produce hyaline membranes in the lungs. Some of the kittens developed membranes resembling those encountered in the lungs of newborn children. Yet in none of the cases could membranes be produced without pneumonia being simultaneously present.

REFERENCES

1. Ahvenainen, E. K.: On the Genesis of Asphyxial Membranes. Symposium on Anoxia of Newborn Infant. London 1951. In press.
2. Pulmonary Hyaline Membranes. Report of the Fifth M & R Pediatric Research Conference, 1952.

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THE MODE OF STROMA FORMATION IN TRANSPLANTED MOUSE CARCINOMA

by

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When carcinoma tissue is transplanted, only part of the epithelial cells survive and, in positive cases, give rise to a growing tumour. The stromal tissue of the graft always undergoes necrosis 2, 7, 5. Thus the stroma necessary for the growth of the transplanted carcinoma is provided by the mesenchyma of the new host. Most earlier writers believed that the connective tissue of the recipient was actively involved in these events. Körbler (4) spoke of an invasion of fibroblasts and a revascularization of the tumour tissue, Rössle of an ingrowth of connective tissue into the young carcinoma tissue. According to Jolles (3), the necrotic stroma is replaced by a new stroma, which adapts itself to the neoplastic parenchyma and rebuilds the original histological architecture.

The manner of stroma formation in both spontaneous and transplanted carcinoma is far from being fully understood. It seems as if too little attention had been given to this question. There is, on the one hand, the well-known destructive influence of infiltrative malignant growth on the surrounding tissues, and, on the other, the selection and incorporation of connective tissue to form an integrating and indispensable part of the tumour itself. In spontaneous carcinoma the relationships between parenchyma and stroma are certainly more complex than in transplantable carcinoma. Observations made on the latter, though of considerable theoretical interest, do not apply directly to spontaneously arising

neoplasms. The properties of transplanted carcinoma will be dealt with in the discussion of the present findings.

Own Investigations. — The relationship between the tumour cells and the host connective tissue in transplanted mouse carcinoma has been studied in order to throw some light on the problem of stroma formation. The investigation was carried out on the transplantable mouse carcinomas Ehrlich (Ref. Cancer 1950:*3:*1052) and Krebs 2 (Ref. Cancer Research 1951:*11:*466); tumour animals were kindly placed at my disposal by Docent G. Klein, M. D., of the Karolinska Institutet, Stockholm. The tumours were implanted subcutaneously into male white stock mice from the Pathological Institute, University of Helsinki. Broadly speaking, 100 per cent of the tumour grafts took.

Since it has been established that infiltrative growth proper becomes discernible on the third day following implantation (4, 7), the experimental animals were sacrificed and the growing tumours were excised from three days to about three weeks after transplantation. Most tumours were examined after three to five days, when the crevice between the graft and the subcutaneous tissue of the host animal, and in particular the interstices between the tissues at either end of the transplant, called by Rössle »Pohlhöhlen» (»polar cavities»), were still partly visible.

The excised tumours were fixed in 10 per cent formol for the purpose of obtaining routine histological preparations; these were stained by van Gieson's method and with haematoxylin and eosin. The material for microscopical investigation was in part fixed in a 4 per cent basic lead acetate solution and subsequently stained with toluidine blue (in 0.5 per cent aqueous solution), and in part fixed in Carnoy's solution. The latter preparations were stained either by the PAS method or by the Azan modification of Mallory's connective tissue stain.

The two types of tumour (Ehrlich and Krebs 2) were used in equal amounts and in the same manner. Since no differences of any consequence in the results were observable between these types, they are dealt with together in the following report.

The most distant, and therefore earliest, reaction observable in the surrounding tissues was intense proliferation of connective tissue. This process was most conspicuous, perhaps, in the muscular and adipose tissues, the muscle fibrils and the fat cells, respectively, being dispersed by the young connective tissue. The latter showed a slight or moderate degree of metachromasia when stained with toluidine blue. Mast cells occurred in it though not in large numbers.

Signs of proliferation in the form of an increased number of nuclei, some of which occupied a central position, soon occurred in the muscle fibrils themselves, too. The muscle fibrils were liable to degenerative changes. As a rule, these were initiated far in advance of the front of the invading tumour tissue. Nevertheless remnants of degenerating muscle fibres were often seen deep in the interior of the neoplastic field.

The cancer cells invading the surrounding tissues behaved differently according to the local conditions and the structure of the tissue in question. In the »polar cavities» and other tissue spaces, which were filled by a serous, often fibrinous fluid, free tumour cells gathered at first mainly along the walls; later they poured out, gradually filling the whole of the interstice. These conditions essentially resemble those in tissue cultures.

When the invading cancer tissue approached soft connective tissue, poor in fibrils, the infiltrative process resembled in many respects that described above. Certain characteristic features were, however, due to the relation of the tumour cells to the connective tissue fibrils. In the interstices of the fibrillary meshwork free carcinoma cells often occurred just as in the »polar cavities», whilst the majority lay in more or less regular rows along the connective tissue fibrils. The structures thus composed of tumour cells and connective tissue resembled berries or grapes on long, slender stalks (Fig. 1, 2). Examination of Azan preparations at high magnification revealed a very close contact between the cancer cells and the host connective tissue fibrils. Although it was sometimes difficult to distinguish young tumour cells from irritated fibroblasts, this contact was clearly visible under favourable conditions. It was found that the cytoplasm of the cancer cell could be almost completely enclosed by an interlacing network of positively staining reticular fibrils, which were sometimes spread over the surface of the carcinoma cell almost like a membrane (Fig. 3, 4). This enfolding of the tumour cells seems to involve active participation by the connective tissue.

It is a very noteworthy fact that no signs of tissue damage occurred in connection with carcinomatous invasion of soft connective tissue of the type here concerned. No disintegrating cells or necrobiotic changes were observable in the connective tissue. The nuclei of the fibroblasts were either normal-looking, oval and

Fig. 1. — Krebs 2 carcinoma. Tumour cells invading loose connective tissue. Carnoy, PAS + haematoxylin × 250.

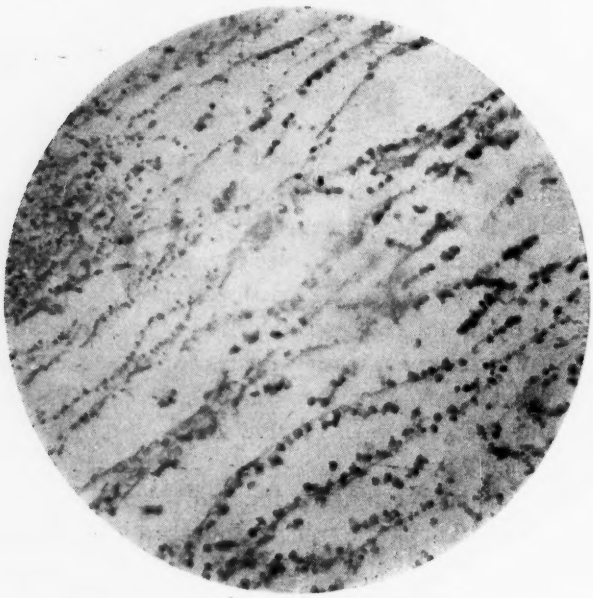

Fig. 2. — Krebs 2 carcinoma. Tumour cells invading loose connective tissue. Basic lead acetate, toluidine blue. × 125.

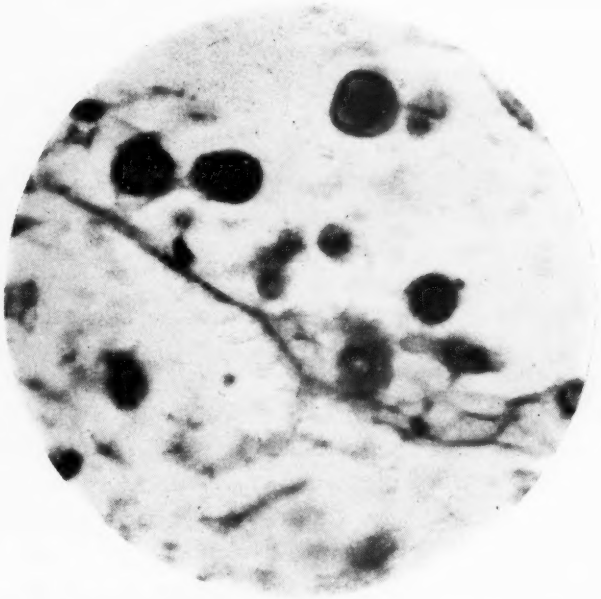

Fig. 3. — Krebs 2 carcinoma. Close contact between tumour cells and connective tissue fibrils. Carnoy, PAS + haematoxylin × 750.

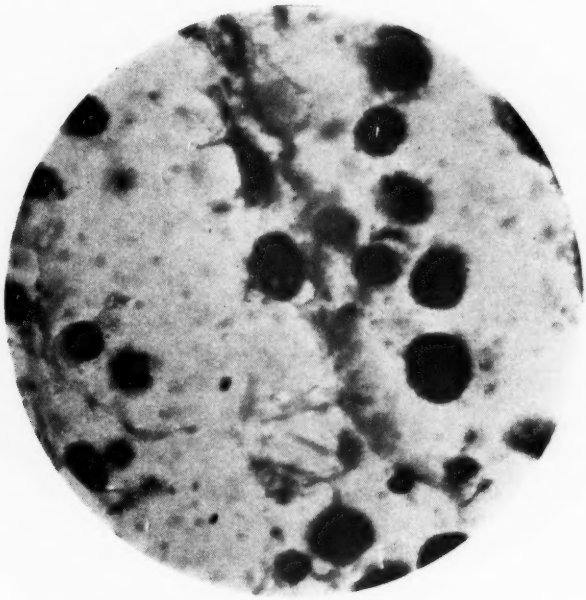

Fig. 4. — Krebs 2 carcinoma. Close contact between tumour cells and connective tissue fibrils. Carnoy, Azan-Mallory. × 750.

fusiform, or they appeared to be somewhat irritated, more rounded and slightly enlarged.

When the connective tissue invaded by carcinoma was dense and rich in fibrils the position was somewhat different. Within a narrow, limited zone at the border between the tumour tissue and the connective tissue signs of tissue degeneration in the form of pyknosis and karyorrhexis were seen. These phenomena did not, however, invariably occur, and they are not of major significance. Degeneration was never extensive, and it is probable that not only connective tissue cells, but tumour cells, too, degenerate.

In dense connective tissue the carcinoma cells also penetrated along the fibrils. As was pointed out above, a small number of fibroblasts and fibrils are perhaps destroyed in such cases, whilst the greater part of the invaded connective tissue persists within the neoplastic field. The same holds true with regard to the capillaries of the connective tissue. The cancer cells migrated in extended rows along the walls of the vessels, which were gradually enfolded by the tumour tissue (Fig. 5). When the cancer tissue approached a field of connective tissue where coarse fibres lay across the path of invasion, this seemed to be barred. It was found that growth by infiltration was slower than the expansion due to cellular proliferation. This may be inferred from the fact that the adjoining connective tissue was pushed aside by the cancer tissue and surrounded concentrically projecting parts of the tumour, as is seen in Fig. 6. The figure also shows a large number of muscle fibrils, arranged as described above, in various stages of degeneration and atrophy.

When a tumour was surrounded by connective tissue rich in fibrils and fibroblasts, this was transformed in the zone of invasion into a relatively scanty framework, which successively changed into the stroma of the fully developed, medullary carcinoma tissue. This stroma consisted of very thin, long fibrils winding between the tumour cells, thus forming a scanty meshwork. Although the fibroblast nuclei were easily distinguishable owing to their narrow, oval shape, it was difficult to discover them in the dense mass of cancer cells. The more rounded fibroblast nuclei were not easily distinguishable from the nuclei of tumour cells. As appears from what has been said above, this rarefaction of the connective tissue is not the result of an active destructive process; it is doubtless due to passive extension of the stromal spongework. Owing

Fig. 5. — Ehrlich carcinoma. Capillaries between rows of invading tumour cells. Carnoy, PAS + hematoxylin. × 750.

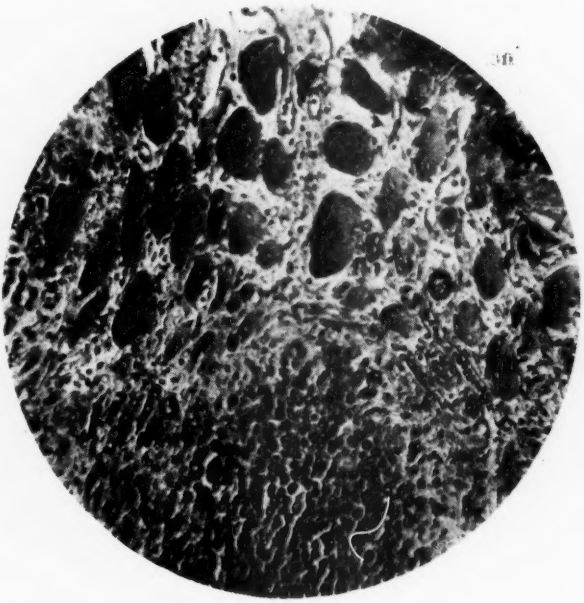

Fig. 6. — Ehrlich carcinoma. Tumour tissue surrounded by concentrically arranged connective tissue and disintegrating muscle fibrils. Carnoy, Azan-Mallory. × 135.

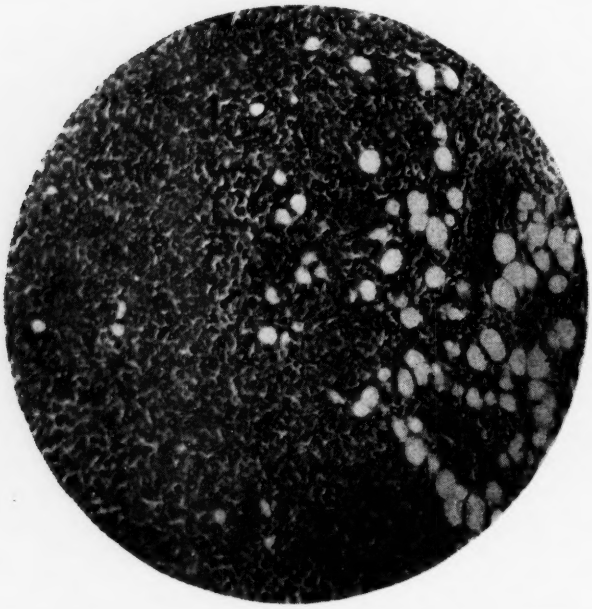

Fig. 7. — Ehrlich carcinoma. Tumour tissue invading subcutaneous adipose tissue. Carnoy, PAS + hematoxylin. × 85.

to a gradual increase in the number of tumour cells in a given amount of connective tissue, the area occupied by this tissue is enlarged. When adipose tissue was invaded by cancerous tissue the result was essentially the same. The fat cells, which are highly resistant to noxious influences, were more dispersed the farther their situation from the margin of the tumour (Fig. 7). Furthermore they occurred in the tumour tissue at a distance from the epidermis exceeding the normal thickness of the subcutaneous adipose tissue. This can only be explained on the assumption that the fat cells are thrust aside in all directions by the multiplying tumour cells.

The initial stage of necrosis of the tumour tissue was in a way the converse of the infiltrative growth. The disintegration and absorption of the devitalized cancer cells resulted in decreased tension and shrinkage of the tumour tissue. Hence the scanty, more or less concealed stromal framework became clearly visible again, but was coarser and considerably denser.

Discussion. — The present observations seem to indicate that, at least as far as concerns the two tumour forms used, the host

connective tissue plays a much more passive part than has previously been assumed. Apart from the proliferation of peripheral connective tissue, no progressive changes were observable indicative of active ingrowth of connective tissue and capillaries into the tumour tissue. In the field of human pathology observations have been reported which seem to indicate that certain malignant tumours »take over» connective tissue without any active participation by this tissue. Böhmig (1), for instance, noted in that advanced epidermoid cancers of the skin subcutaneous connective tissue was walled in by rows of tumour cells. He reported a similar situation in breast carcinoma, where the soft interlobular connective tissue was directly transformed into neoplastic stroma. The partial agreement between the present observations and those relating to certain malignant tumours in man are not, however, evidence of any essential resemblance between transplantable and spontaneous carcinomas. One of the main differences is that the former do not, as a rule, metastasize. This is undoubtedly contingent on the fact that the connective tissue is hardly, if at all, destroyed, as was established in the present investigation. Furthermore it was shown by Santesson (8) that, *in vitro*, spontaneous epithelial mouse tumours possess the capacity to dissolve the plasma fibrin and form »crescents», and to dissolve the fibrillary structures formed by the connective tissue cells. Transplanted mouse tumours lack this capacity.

The destructive action at a distance upon surrounding tissues usually characterizing malignant tumours, which was described by Sylvén (9) with regard to mesenchymal tumours, was clearly observable in my series as far as muscular tissue was concerned. In contrast to this, the almost absolute resistance of the connective tissue was the more conspicuous.

The close contact established in fields of soft connective tissue, in particular, between this and the cancer cells deserves of special attention. The examination of such fields gives the impression that direct nutrition by the connective tissue must be involved. This assumption is in good agreement with observations showing that transplanted tumour tissue is dependent on the properties and the state of the host connective tissue. In this connection the reader is referred only to Cramer's (2) interesting observations on radioresistant and radiosensitive breast carcinoma in mice and to the

investigations of Oughterson *et al.* (6) on cure rates after irradiation in different strains of mice. The significance of the stromal reaction in radiational therapy is discussed at length in Jolles's monograph of 1953. The fact that homologous grafts do not take in a bed previously exposed to an erythema dose of X-rays is also clear evidence of the intimate relationship between cancer cells and connective tissue. It seems as if the direct destructive effect on the tumour cells had been too greatly stressed in the literature dealing with the effect of radiation on malignant tumours.

SUMMARY

The mode of stroma formation in growing tumours was investigated on Ehrlich's and Krebs's carcinomas, transplanted subcutaneously into male white stock mice. Reactive proliferation of the surrounding connective tissue and early degenerative changes of the muscular tissue were observed.

The cancer cells migrated along the walls of the crevices between the graft and the surrounding tissues. Later these spaces were gradually filled by free, outpouring tumour cells.

In soft connective tissue the invasion did not cause any observable damage. Some cancer cells were dispersed in the tissue spaces, whilst the majority arranged themselves along the connective tissue fibrils. A close contact with these was established and appeared to involve direct nutrition by the connective tissue.

In dense connective tissue, rich in fibrils, signs of tissue damage in the form of pyknosis and karyorrhexis were occasionally observed at the margin of the tumour tissue. The main part of the invaded connective tissue and the capillaries in this area remained within the neoplastic field, where it formed a very thin, scanty stromal network, highly distended by the multiplying cancer cells. As necrosis of the tumour tissue set in, this network again became clearly visible; it was now coarser and denser owing to the relaxation of the tissue.

REFERENCES

1. Böhmig, R.: Beitr. path. Anat. 1930:*83:*333.
2. Cramer, C.: Zschr. Krebsforsch. 1928:*26:*194.
3. Jolles, B.: X-ray Sieve Therapy in Cancer. A Connective Tissue Problem. London 1953. H. K. Lewis.
4. Körbler, J.: Zschr. Krebsforsch. 1936:*36:*572.
5. Oberling, Ch.: The Riddle of Cancer. New Haven 1944. Yale Univ. Press.
6. Oughterson, Tennant and Lawrence: cit. by Jolles.
7. Rössle, R.: Sitzungsber. d. Preuss. Akad. d. Wissensch., Phys.-mathem. Klasse 1936:22.
8. Santesson, L.: Acta Path. Microbiol. Scand. 1935, Suppl. 24.
9. Sylvén, B.: Acta Radiolog. 1945, Suppl. 59.